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LETTER FROM THE EDITORS

Dear Friends and Colleagues of the Vanderbilt Neuroscience Community,

It is our pleasure to bring you another exciting issue of the *Vanderbilt Reviews Neuroscience*. This is the sixth volume of the *VRN* and contains the largest number of candidate reviews to date, demonstrating a successful future of the Vanderbilt Neuroscience Program. In this issue you will find seventeen skillfully crafted reviews encompassing the diverse research interests of our newest Ph.D. candidates. In addition to our growing student body and breadth of research topics, we can also pride ourselves on the quality of the research being done here in the Neuroscience Program. In the last calendar year, several of our students have published first-author papers in high-impact journals across multiple disciplines of neuroscience. In sticking with tradition, this *VRN* issue highlights just a few of these publications in the Research Highlights and Research Briefs sections. You will also find information regarding some of the wonderful outreach programs supported by the Vanderbilt Brain Institute, including our annual Brain Blast event and our first Music & the Mind Symposium. We also thank Britney N. Lizama-Manibusan for her beautiful cover image. You can read more about Britney's project and the *VRN* cover image in the On the Cover section.

We would like to thank the *VRN* editing team for their hard work and enthusiasm in preparing this issue for publication. Our co-editors Kale Edmiston, Terry Jo Bichell and Amy Palubinsky were dedicated, enthusiastic and a joy to work with- thank you so much! Additionally, we thank the previous *VRN* editors, Sudipta Chakraborty and Juliane Krueger for all their advice and resources needed to assemble this issue. We thank Drs. Mark Wallace and Bruce Carter for their support of the student-run *VRN* and for maintaining the high level of training in the Vanderbilt Neuroscience Program. Most importantly, we would like to thank the 2013 qualifying class for their time and patience in generating this issue. It was a pleasure getting to know you all and learning about the interesting work you will do.

Your Co-Editors-in-Chief,

Barbara O'Brien and Tyne Miller-Fleming

From left: Barbara O'Brien, Amy Palubinsky, Terry Jo Bichell, Tyne Miller-Fleming, and Kale Edmiston

Barbara O'Brien and Tyne Miller-Fleming

photos taken by Lakshmi Sundararajan

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Vanderbilt Reviews Neuroscience(VRN) is an open-access journal. *VRN* is the official journal of the Vanderbilt University Neuroscience Graduate Program and the Vanderbilt Brain Institute. *VRN* is a collection of reviews submitted by Vanderbilt Neuroscience Graduate Students whilst qualifying for doctoral candidacy. The journal also offers highlights and commentary on work being done at Vanderbilt and Neuroscience laboratories around the world. *VRN* was founded in 2009 in an effort to consolidate and recognize the hard work done by each class of Ph.D. qualifiers, and is published annually by the Institute.

Review Process
All reviews submitted for doctoral qualifiication must be approved by a committee of at least four tenured or tenure-track faculty members (Phase I). All approved reviews are accepted by *VRN*.

A Message from the Director of the Vanderbilt Brain Institute

It seems only yesterday that we were publishing the first issue of *VRN*. An experiment at the time, I am very impressed that the journal has endured and am very proud when I take it with me to other institutions to show off the quality and diversity of the research performed by our students. The reaction is a pretty universal "Wow – what a fantastic idea and what an impressive body of work."

I also marvel at our discipline and the remarkable conceptual and technical advances that continue to catapult neuroscience research forward at a dizzying pace. Advances at the molecular level such as CRISPR-Cas are revolutionizing our ability to edit and regulate genes and genomes, and hold remarkable promise in the therapeutic arena. At the other end of the spectrum, but also with tremendous translational and clinical relevance, are more systems-based approaches such as deep brain stimulation (DBS) and transcranial direct current stimulation (tDCS). We are very fortunate to be at a time and place where we can see and use these tools that were only a short while ago the realm of science fiction, and perhaps more importantly see the promise they hold for bettering the human condition – particularly as they relate to neurological disease and mental illness.

Yours in science,

Mark T. Wallace

A Message from the Neuroscience Program Director of Graduate Studies

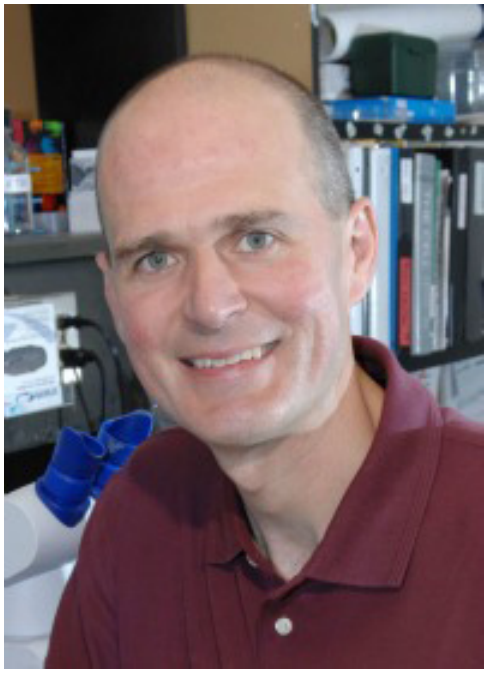
Dear Readers,

It is with great enthusiasm that I take on the mantle of Director of Graduate Studies (DGS) for the Neuroscience program! I realize that I have some big shoes to fill with Doug McMahon stepping down after leading the program to be ranked as one of the top neuroscience graduate programs in the country. Under Doug's guidance, our program was awarded the Neuroscience Program of the Year in 2012 by the Society for Neuroscience, the student body has grown to become one of the largest graduate programs at Vanderbilt and our training paths were unified so that the two previous tracts (Cellular & Molecular and Systems) are now one. Most importantly, he always demonstrated (and still does) a deep commitment to the education of our students in the classroom, the laboratory and as future leaders in all aspects of science. On behalf of all members of the Vanderbilt Brain Institute, let me take this opportunity to thank Doug for all he has done.

As the new DGS, I commit to doing my best to follow the example set by Doug, and it is with great humility and appreciation that I take on this role. It is my vision to work with our Director, Mark Wallace, and the other leadership team members to continue to strengthen our program, even during these difficult financial times. In my opinion, any educational program must never be satisified with the status quo and must constantly and unrelentingly seek to improve itself. It is my intention to take advantage of our most valuable asset, the students themselves, in seeking ways that we can continue to develop. Our students are among the brightest, most creative and innovative young minds in the world, and I cannot imagine a better resource for designing new educational opportunities and growing our program in all ways. I see my role as the axon hillock, gathering as many inputs as possible and deciding when to fire the action potential of change. My door is always open, and I welcome hearing from each of you!

Sincerely,

Bruce Carter



An Update from the Neuroscience Student Organization President

It has been a privilege to serve as the Neuroscience Student Organization (NSO) president for the past year. I would like to take this opportunity to welcome new students to the neuroscience graduate program. I encourage each of you to reach out to more senior students and faculty as you navigate your first year of graduate school; we are here to help support you. I would also like to commend our seventeen new PhD candidates, whose qualifying review papers are published in this journal thanks to the hard work of *Vanderbilt Reviews Neuroscience* editors Barbara O'Brien, Tyne Miller, Terry Jo Bichell, and Amy Palubinsky.

The purpose of the NSO is to improve student quality of life with social events and academic support and by facilitating student communication with program administration. NSO members have been hard at work this year. Daniel Bermingham and Kelli Money revamped and organized the third annual boot camp, which helps new students prepare for their coursework. Academic committee members Courtney Bricker, Le-Anne Kurela, and Elaine Ritter have expanded and improved upon the study session and mock qualifying exam model for students preparing for the first phase of their candidacy exams. Unsurprisingly, Tristan Watkins and Pratik Talati have planned spectacular social events that have facilitated relationships between students in different labs and areas of study. I encourage all students to get involved with service to the program as a way to ensure that the NSO and VBI reflect our interests.

The NSO recently hosted our 17th annual program retreat. The retreat is a time to relax and catch up with other program members and get to know the new students. Thanks to the efforts of Eddie Hickman, Emily Mason, and Tyne Miller, the retreat was held at the Nashville Zoo and was a great success. We hope you visited the wombats between whatever immunoassays or MRI scans you might have been conducting that week.

Vanderbilt Neuroscience has continued to model outstanding community engagement. This year marked another successful Brain Blast event, as well as the addition of a number of other public talks, thanks to the hard work of Victoria Cavener and Emily Mason. VBI faculty Dr. Paul Newhouse and Dr. Beth Malow gave a well-attended presentation for the lay public in the spring for the Jeannette Norden Outreach Lecture "Building a Healthy Brain." Dr. David Zald and psychology faculty Dr. Marianne Ploger recently presented at the Music and Mind Symposium with Daniel Levitin and Ben Folds, discussing ways in which neuroscience and music inform each other.

This year has certainly had its challenges as funding for scientific research nationally has continued to shrink. Despite this, Vanderbilt continues to lead the nation in research funding and innovation. I am impressed by the way in which VBI staff, students, and faculty have risen to the occasion, not only making the best of challenging situations, but using them as opportunities to improve our program and emphasize the importance of creative problem solving when budgets are tight. I would in particular like to highlight the contributions of our incredible administrative staff: Rosalind Johnson, Mary Michael-Woolman, Denise Malone and Beth Sims. Their dedication to the program has made each of our successes possible- from grant submissions and conference presentations to the day-to-day organization that keeps our program running. I encourage everyone to take the time to thank the VBI staff for their hard work.

E. Kale Edmiston

Brain Blast & Community Outreach

In a 2007 editorial in Science, Alan Leshner, former CEO of AAAS, discussed the need for graduate students to be trained in the communication of science to the lay public. Said Leshner, "If science is going to fully serve its societal mission in the future, we need to both encourage and equip the next generation of scientists to effectively engage with the broader society in which we work and live."

The mission of the Community Outreach Committee of the Neuroscience Student Organization is to provide opportunities for neuroscience students to engage with the public through lectures, fundraising, and education.

Each year, Vanderbilt neuroscience students invite one speaker whose research is both exciting and innovative. This year, the invited speaker was Dr. Ken McCarthy of the University of North Carolina, Chapel Hill. Dr. McCarthy spoke to a packed house about his research on the vital role that astrocytes play in neural processes. Dr. McCarthy was also able to speak with students individually throughout his visit. He shared his love of mentorship, and spoke often about how he had the "best job in the world." Dr. McCarthy's enthusiasm was infectious.

One of the most exciting programs hosted by the NSO is Brain Blast, a neuroscience education event designed for children. This day-long event was held last March at One Hundred Oaks and featured educational booths run by student and faculty volunteers. Over 250 children and their families took part in Brain Blast. There were over 20 booths where children were able to extract DNA, learn about optical illusions, practice mind control, and so much more! Children and families were also welcome to take tours of the Brain Matters exhibit. As well as being a great way to engage with the community, Brain Blast is an opportunity to meet neuroscientists from outside of Vanderbilt. This year there were over 80 volunteers from Vanderbilt, Middle Tennessee State University, and Tennessee State University. This event was a huge hit, and there was a lot of great feedback from children, parents, and volunteers.

The Community Outreach Committee is planning future activities so stay tuned!

Emily Mason
Eddie Hickman
Victoria Cavener

Left: Children visit an exhibit to teach them about sensory systems. Above: Participants extract DNA from strawberries and learn about how the nervous system works.

A Message From Your Middle Tennessee SfN Chapter

Dear Members of the Vanderbilt Neuroscience Community,

I am writing to update you on another successful year for the Middle Tennessee Chapter of the Society for Neuroscience (MTNCSfN). The "mission" of our chapter is to unite neuroscience in Nashville and the surrounding area, with various activities designed to promote the exchange of ideas between neuroscientists at all levels and to inform the public about brain science. As such we continue to expand our membership both at Vanderbilt and several other local institutions including Meharry, Belmont, Tennessee Tech, Austin Peay, Fisk and TSU.

Our annual Fall social and Halloween party was held at the end of October 2013 in conjunction with the Neuroscience Student Organization. In addition to enjoying food, drinks, and catching-up with colleagues, the election results for the new chapter officers were announced. Our contact information can be found on the chapter website (http://www.mtncsfn.org/), and we would love to hear from you if you have any ideas for chapter activities or want to become more actively involved.

Held in May, our annual chapter meeting featured faculty members Brian Nelms (Fisk), Akiko Shimamoto (Meharry), and Alex Maier (Vanderbilt) who treated us to short talks about their research. Even more entertaining were the trainee data-blitz competitions, in which grad students or post-docs presented a 3-minute synopsis of their latest research. Everyone did an amazing job, but the audience voted, and our $50 prize winners were Max Joffe and Jennifer Walker. A reception with snacks and drinks featuring Bruce's home brew concluded a fun meeting.

Each year the Chapter can nominate one graduate student and one post-doctoral fellow who go on to compete at the national level for travel awards to attend the annual SfN meeting. This year we had a great pool of applicants, and from these we selected Sarah Baum (post doc in Wallace lab) and Pooja Balaram (grad student in Kaas lab) to be our 2014 nominees– congratulations! Also congratulations to our nominees from last year, Devon Graham and Kevin Kumar, who both won travel awards to attend SfN 2013 in San Diego! We were also successful in winning a Chapter grant from SfN to help support our Summer Enrichment Research Program in Education and Neuroscience Training, or "SERPENT". This provides support for an undergraduate from a local institution to work in a Vanderbilt Neuroscience lab and participate in the Vanderbilt summer science academy. The SERPENT student also develops educational or outreach material suitable for the general public and K-12 students. This is presented at the VBI sponsored Brain Blast event held each March to kick-off brain awareness month. You can check out some of the outreach material developed in previous years by following the "resources" link on our website. This past summer we welcomed Kathryn Hook from Belmont University who worked in BethAnn McLaughlin's lab.

If you are not already a member, I hope you will consider joining and help us become a showcase example of what can be done through the local chapters. Visit our website to learn more (http://www.mtncsfn.org/), and you will be just one click away from joining and/or making a donation!

Sincerely,

Kevin Currie

President, Middle Tennessee Chapter of the Society for Neuroscience

Inaugural Vanderbilt Music and Mind Symposium 2014

The Vanderbilt Brain Institute presented the inaugural "Music and Mind Symposium: Exploring Novel Connections between Neuroscience, Psychology, and Music" on June 12, 2014. The evening program, held at Ingram Hall at the Blair School of Music, combined research, community, performance, presentation, and panel discussion. The goal of the event was to engage the Vanderbilt academic community, those in the Nashville music industry, and the public in a discussion about the connections between neuroscience and music. Capacity at Ingram Hall is 600, and the event sold out in three days.

The free event was composed of two parts: a Research/Community Exposition followed by a Performance/Discussion Event. The Research/Community Exposition featured 16 posters from Vanderbilt researchers currently involved in brain/music research and/or education. Local and national organizations interested in brain/music research also participated as community partners in the first portion of the evening and hosted tables featuring information about their organizations. The GRAMMY Foundation, MusiCares, Musicians On Call, and NAMI were among 10 exposition community partners. Catering and beverage services were kindly provided by local vendors, including Yazoo Brewing Company.

The Performance/Discussion portion of the evening included Ben Folds, multi-platinum selling singer/songwriter/producer, and Daniel J. Levitin, Ph.D., James McGill Professor of Psychology, Behavioral Neuroscience, and Music at McGill University. Two Vanderbilt faculty members, Marianne Ploger, Director of the Musicianship Program at the Blair School of Music, and David Zald, Ph.D., Professor of Psychology and Psychiatry, later joined Mr. Folds and Dr. Levitin on the stage for further discussion and an audience Q&A session.

Vanderbilt academic heads Mark Wallace, Ph.D., Director of the Vanderbilt Brain Institute, and Elizabeth Dykens, Ph.D, Director of the Vanderbilt Kennedy Center, were among those in attendance. Also in attendance were music industry leaders Jed Hilly, Executive Director of the Americana Music Association, and Craig Havighurst, Producer of Music City Roots, as well as noted and critically acclaimed artists Kenny Malone and Darrell Scott.

Dr. Wallace shared his take on the event: "The first annual Music and the Mind event at Vanderbilt exceeded our wildest expectations! The engagement and discussion between the scientific and artistic communities at the research expo was stimulating and thought-provoking, and the feature event was an expansive and enlightening conversation between Ben Folds, Dan Levitin and two Vanderbilt faculty with unique perspectives on music and the brain. We continue to ride the wave of enthusiasm that stemmed from the event, and that has forged remarkable intersections between the neuroscience and music communities."

The second Vanderbilt Music and Mind event will be part of the Society for Music Perception and Cognition symposium, hosted by Vanderbilt next summer. Event participants are to be announced.

Nicole L. Baganz, Ph.D.

The Vanderbilt Brain Institute hosted the first Music and Mind Symposium 2014. From left to right: Daniel Levitin, Ben Folds, David Zald, and Marianne Ploger.

RESEARCH HIGHLIGHTS

Changes in Ocular Pressure: Not a Fun Trip When You're Missing TRPV1

Glaucoma is the leading cause of irreversible blindness throughout the world. An optic neuropathy, this devastating disease involves the degeneration of retinal ganglion cells (RGCs), which express a family of cation-selective channels known as transient receptor potential (TRP) channels. TRP channels are extremely diverse in their function and response to both physiological and pathogenic stimuli based on the subunits they express. One particular subunit, capsaicin-sensitive TRP vanilloid-1 (TRPV1) responds to changes in intraocular pressure (IOP), to which patients with glaucoma are particularly sensitive.

In a recent article in the Journal of Neuroscience, graduate student Nick Ward and colleagues used a single, calculated injection of microbeads into the anterior portion of the eye to induce a chronic, but moderate increase in IOP. Using this method in TRPV1 knockout (KO) mice and C57 control animals, the authors found that loss of this channel had a severe detrimental effect on anterograde axonal transport to the lateral geniculate nucleus (LGN) and a nearly complete loss of transport to the superior colliculus (SC). Previously, disruptions in transport to the SC and LGN have been demonstrated to be an early sign of RGC axon dysfunction within the optic projection. Upon further evaluation, the authors discovered that TRPV1 KO also results in a significant acceleration of pathology when IOP is elevated as indicated by: degenerating axons, decreased axon density, smaller axon bundles and an overall decrease in axon numbers. Similar results were found using pharmacological antagonism of TRPV1 in a rat model of increased IOP via the same microbead injection paradigm.

To further understand the mechanisms behind the accelerated degeneration of RGCs in response to elevated IOP, the authors utilized single cell patch clamp techniques. RGCs were separated into two groups for analysis: those with relatively high spontaneous firing rates (3-15 Hz) and those with low spontaneous rates that required injection of current to exceed a threshold firing rate of 3 Hz. In C57 mice, RGCs exhibited increased spontaneous firing rates in response to elevated IOP. Interestingly, this increase was abolished in RGCs from TRPV1 KO animals. For RGCs with low firing rates, it was determined that, in response to elevated IOP, greater depolarizing current was needed to reach the threshold firing rates in TRPV1 KO animals. Altogether, these results indicate that TRPV1 KO causes RGCs to lose an intrinsic mechanism that enhances RGC excitation in response to stressors.

Strikingly, the absence of TRPV1 alone does not induce neurodegeneration. Both C57 and TRPV1 KO animals exhibited pathology only in response to IOP elevation; however, this pathological progression was accelerated in TRPV1 KO mice. Likewise, the physiological response of RGCs to elevated IOP was altered by TRPV1 KO. Together, these results suggest that TRPV1 helps counter neuronal stressors and plays a major role in the survival of RGCs. Due to the careful and controlled nature of this study, the authors have discovered novel molecular mechanisms of axonal degeneration that may be further leveraged to identify therapeutic targets for the millions of patients affected by glaucoma as well as other neurodegenerative disorders.

Nicholas J Ward, Ho, KW, Lambert, WS, Weitlauf C, & Calkins, DJ (2014). Absence of transient receptor potential vanilloid-1 accelerates stress-induced axonopathy in the optic projection, *J Neurosci* 34(9):3161-70.

A novel PIP2 interaction with the dopamine transporter regulates dopamine efflux

Phosphatidylinositol 4,5-bisphosphate, or PIP2 is a component of cell membranes with established roles in cellular metabolism and signaling.Previous reports suggest that PIP2 may play a more diverse role than previously imagined by forming interactions with membrane proteins, including the serotonin transporter, SERT. In this paper Hamilton et. al. describe a novel role for PIP2 as a regulator of dopamine efflux. The authors use a variety of biochemical and electrophysiological assays to confirm a direct interaction between the dopamine transporter (DAT) and PIP2, describing a new regulator of dopamine-dependent behaviors.

Hamilton et al. used fluorescence microscopy and co-immunoprecipitation to show that GFP-tagged DAT and PIP2 interact in hDAT cells, CHO cells transgenically expressing the human DAT. These studies were repeated in preparations of mouse striatum to confirm that this interaction is present in brain tissue. To test for a direct interaction, the authors generated an N-terminal fragment of DAT and tested its binding efficiency in liposome mixtures containing PIP2. Indeed, the N-terminal region of DAT directly binds the PIP2 liposomes. The N-terminal fragment used in these experiments contained 64 amino acids and the authors

wanted to better characterize the specific amino acids that facilitate this interaction. Using computational modeling and more liposome biochemical assays, the authors show that two lysine residues (Lys3 and Lys5) near the N-terminus of the DAT protein mediate its electrostatic interactions with PIP2. When the authors mutate these sites (hDAT K/A), the interaction with PIP2 is lost.

Identification of this novel PIP2-DAT interaction was exciting, but the authors next wanted to understand the functional implications of this association. The authors used amperometry to measure dopamine efflux from hDAT cells. Amperometry is a technique used to detect the concentration of molecules that can be oxidized (in this case, dopamine) by measuring electrical current changes. Specifically, in these experiments, the authors are able to measure dopamine efflux with an amperometric electrode placed very close to the hDAT cell membrane. Coincidentally, they patched the hDAT cells with another electrode to maintain the desired levels of intracellular dopamine and deliver specific molecules. In wild-type cells, administration of amphetamine (AMPH) causes the efflux of intracellular dopamine through the DAT. Interestingly, when the authors block the PIP2-DAT interaction or deplete PIP2, this AMPH-driven dopamine efflux is diminished. The authors also find that the PIP2-DAT interaction is not required for influx of amphetamine or dopamine through the DAT, suggesting this interaction is specifically required for dopamine efflux.

The authors complete their story by showing this interaction is conserved in living organisms and has behavioral consequences. In *Drosophila* locomotion defects have been used to identify regulators of dopamine signaling; for example, flies expressing mutant DAT protein are hyperactive. The authors are able to rescue this hyperactive phenotype by transgenic expression of hDAT and hDAT K/A. AMPH causes hyperactivity in wild type animals, which the authors can recapitulate in flies expressing hDAT. When the hDAT K/A animals are treated with AMPH, the locomotor response is diminished, suggesting that the PIP2-DAT interaction is required for AMPH-driven hyperactivity in *Drosophila*. The authors confirm these results with amperometry measurements in cultured *Drosophila* neurons. This work provides a new and exciting role for PIP2 as a regulator of dopamine-dependent behaviors, and raises the possibility that PIP2 may regulate other transporters and ion channels that control complex behaviors across phylogeny.

Peter J Hamilton, Belovich, AN, Khelashvili, G, Saunders, C, Erreger, K, Javitch, JA, Sitte, HH, Weinstein, H, Matthies, HJG, & Galli, A (2014). PIP2 regulates psychostimulant behaviors through its interaction with a membrane protein. *Nature Chemical Biology* 10(7):582-9.

New Spot for Pot: Endocannabinoids in the Central Amygdala

The central amygdala (CeA) plays an essential role in response to anxiety and fear. More reecently, it has been suggested that endocannabinoid (eCB) signaling may play a role in such responses. However, how eCBs modulate this circuitry has been understudied due to lack of effective tools. In a newly published article, Ramikie and colleagues sought to uncover the synaptic mechanisms inherent to this region to provide added insight into the mechanisms that regulate fear and anxiety. Utilizing a number of tools, this group demonstrated abundant expression of eCB signaling elements specifically at glutamatergic synapses of the CeA that facilitate several mechanistically and temporally distinct modes of postsynaptic eCB mobilization.

This group also determined that endocannabinoid type-1 receptor (CB1) positive afferents form glutamatergic synapses onto CeA neurons. In addition, they found that these same excitatory synapses express the enzyme, DAGLα, which is responsible for synthesizing 2-AG, one of the major eCBs in the CNS.

Using impeccably designed electrophysiological experiments, the authors sought to understand the functional significance of CB1 expression within the CeA. Initial experimental analyses indicated that CB1 receptors mainly function to regulate glutamatergic transmission, via suppression of glutamate release. Additional experiments next revealed that eCB mobilization at these glutamatergic synapses can be mediated by 2-AG activation of CB1 receptors.

Given that eCB mobilization can also be mediated by G-protein-coupled receptors, the authors examined muscarinic acetylcholine receptors (mAChRs) and found that these receptors are not only present, but like the CB1 receptors, are also functional within the CeA. Interestingly, the authors found that synaptic depression is only partially CB1-dependent and that G_q-receptor-driven eCB mobilization can also be initiated within the CeA to modulate glutamatergic transmission.

An elegant set of pharmacological experiments allowed the authors to temporally delineate between the signaling that occurs at each receptor type. They found that AEA, rather than 2-AG, is is the eCB ligand that acts acutely

on CB1 receptors to reduce glutamate release. While prolonged stimulation of mAChRs causes CB1-mediated synaptic depression via the release of 2-AG through the canonical calcium-DAGL-dependent pathway. Interestingly, this same temporal phenomenon of mAChR-driven multimodal eCB release is not generalizable as examination of these pathways in the striatum did not yield the same results, suggesting region- and/or cell-type specific signaling mechanisms.

Overall, these data indicate that CeA neurons can utilize multiple types of eCB signaling to modulate afferent glutamatergic transmission and that AEA and 2-AG can be differentially released in response to activation of the same G_q-coupled receptor, depending on the duration of the stimulus. Continued investigation of these signaling pathways is extremely important as it can provide insight into the synaptic mechanisms regulating stress response physiology, anxiety-like behaviors and emotional learning.

Teniel S Ramikie, Nyilas, R, Bluett, RJ, Gamble-George, JC, Hartley, ND, Mackie, K, Watanabe, M, Katona, I, & Patel, S (2014). Multiple mechanistically distinct modes of endocannabinoid mobilization at central amygdala glutamatergic synapses, *Neuron* 81(5):1111-25.

Affecting Affective Behavior: GluN2B Subunits in the BNST

Anxiety disorders and depression are among the most common mental disorders throughout the world affecting roughly 1 in 10 persons. Although it is estimated that 121 million people struggle with these disorders, less than 1/3 are receiving adequate treatment. Promising new therapeutics include N-methyl D-aspartate receptor (NMDAR) antagonists; however, these drugs can mimic the symptoms of psychosis, have high abuse potentials and have off-target effects. Therefore, careful analysis of the mechanisms by which this class of drugs acts can lead to the development of safer therapeutic options. Initial studies in humans and rodents demonstrated reduced anxiety following a single administration of the global NMDAR antagonist, ketamine as well as an antagonist specific to the GluN2B subunit of NMDA receptors (Ro 25–6981). Given that the bed nucleus of the stria terminalis (BNST) has been implicated in depression and anxiety and that it contains high levels of GluN2B expression, Louderback and colleagues hypothesized that NMDARs within this brain region may provide significant contribution to the antidepressant actions of NMDAR antagonists.

In order to test this hypothesis, the authors first validated the use of a novelty-induced hypophagic (NIH) paradigm often utilized in chronic stress studies, within an acute setting. In brief, mice were restrained for 60 minutes (to cause acute stress) or unrestrained. Thirty minutes later, the mice were divided into 3 groups and administered either ketamine, Ro 25–6981 or saline. Following injection, the mice were then placed into a novel cage equipped with a highly palatable food (vanilla flavored Ensure) and the latency to consume Ensure was quantified as a measure of the affective state. Important to note is that the mice in this study had access to normal chow and all of the mice consumed similar amounts of Ensure during training, therefore the results obtained from this task are based on the animals' hedonic drive and not hunger. Using this novel paradigm, the authors found that animals given ketamine or Ro 25–6981 had a significantly reduced latency to consume Ensure whether unrestrained or restrained as compared to those administered saline, suggesting a decrease in depression-like behavior. Interestingly, using other common behavioral tasks, such as forced swim followed by analysis via the elevated zero maze, no changes were seen in distance traveled or time spent in the open arm with ketamine or Ro 25–6981 administration, not only validating this paradigm, but demonstrating it as a superior model for such studies.

The authors next moved on to discerning which specific neural circuits are involved. To do this, the lab generated mice with knock down of the GluN2B subunit within neurons of the BNST via precise injection of a lentiviral vector into this region and found that these animals display similar responses to those administered ketamine or Ro 25–6981. Together with previous studies demonstrating that knockdown of GluN2B in corticohippocampal regions had no effect on affective behavior, these results clearly implicate GluN2B subunits specifically within the BNST in reducing negative affective behavior and may represent a novel target for therapeutics.

Katherine M Louderback, Wills, TA, Muglia, LJ, & Winder, DG (2013). Knockdown of BNST GluN2B-containing NMDA receptors mimics the actions of ketamine on novelty-induced hypophagia, *Transl Psychiatry* 3:e331.

Large Scale Brain Networks Work Together During Memory Retrieval

The default mode network (DMN) and dorsal attention network (DAN) are anti-correlated brain networks

that have been described using functional connectivity magnetic resonance imaging (fcMRI) methods. The DMN consists of cortical regions (i.e., the posterior parietal cortex, hippocampus, posterior cingulate cortex, and the medial prefrontal cortex) that have correlated blood oxygen dependent (BOLD) signal during internally directed cognitive states. The DAN is composed of the dorsal prefrontal cortex, particularly the frontal eye fields, and the intraparietal sulcus, and is thought to mediate attention-based orienting that is mediated by external, environmental cues. In a recent paper by James Kragel, a neuroscience graduate student in the Polyn Lab, interactions between the DMN and the DAN were investigated using independent components analysis (ICA), a computational methodology used to examine and separate the contributions of cortical networks to an overall BOLD signal pattern during a task or at rest.

In this study, participants underwent an fMRI scan and completed a free-recall paradigm, which asked participants to study and encode lists of words and then recall them in the scanner. The authors identified subcomponents of the DMN that varied in their engagement throughout the free-recall portion of the task. However, a subcomponent termed the posteriomedial network, consisting of the ventromedial prefrontal cortex, retrosplenial cortex, and bilateral temporal cortex, was the only sub-network demonstrating sustained, increased engagement throughout the free recall portion of the task. Furthermore, correlational analyses revealed changes in functional coupling between subcomponents of the DMN and DAN that were dependent on task phase and performance. These findings suggest that large-scale functional networks are likely supported by contributions of a number of sub-networks that can function both cooperatively or competitively to facilitate free recall. These findings also challenge the prevailing notion that the DMN and DAN are recruited solely in opposition to each other, and suggest a much more flexible and heterogeneous system of neural networks that facilitate cognition.

James E. Kragel & Polyn, SM (2013). Functional Interactions Between Large-Scale Networks During Memory Search, *Cerebral Cortex* 10.1093/cercor/bht258.

Size Doesn't Matter: Understanding Anxiety and Addiction through Imaging of the 190mm³ BNST

Anxiety is a feeling of dread or apprehension about a future event, distinct from the feeling of fear, which is connected to a real and present danger. Although it is possible for anxiety to be appropriate, increasing vigilance in potentially threatening situations, it is often misplaced and when sustained can lead to an anxiety disorder, which is one of the most common and debilitating mental illnesses. Chronic anxiety is associated with drug addiction and interestingly, both disorders seem to be mediated by a tiny nucleus in the extended amygdala, the Bed Nucleus of the Stria Terminalis (BNST), which is also involved in reward-seeking behavior. Optogenetic studies in rodents have demonstrated that functional circuits, connecting through the BNST, are more important in the mediation of these complex behaviors than structural connections may be. Elucidating the functional connectivity of the BNST could lead to better understanding of the neural mechanisms underlying both anxiety and addiction.

Most studies of BNST neurocircuitry have used luminescent tracers in rodents, revealing extensive connections between the BNST and both limbic and striatal regions. Though interesting, these results may not completely explain the connectivity of this important nucleus in humans, because the primate BNST is larger relative to adjacent structures than it is in rodents, suggesting a more extensive neurocircuitry. Studies of primates have explored connectivity between the BNST and other parts of the limbic structures, but none have examined BNST connections to the rest of the brain. A recent paper from the Blackford lab, uses new high-resolution imaging methods to explore neurocircuitry between the BNST and the rest of the human brain, identifying two novel connections which were previously unknown, and outlining the key nodes of the human BNST circuit.

One of the major benefits of high-resolution imaging is that it makes it possible to examine neurocircuitry *in vivo* in awake human subjects. The current paper uses both Diffusion Tensor Imaging (DTI) and Resting State Functional Magnetic Resonance Imaging (rs-fMRI) to identify the structural and functional connections between the BNST and the rest of the human brain. DTI is a technique that takes advantage of the directional diffusion of water in tissue to reveal neural tracts, while rs-fMRI collects information about regional interactions that occur without conscious activity. This study includes a large sample size, with 82 DTI's and 99 rs-fMRIs, from a total of 120 healthy participants between the ages of 17 and 57, of which 47.7% were female. Subjects first received a T1-weighted scan to determine gross regional brain structure, followed by seven minutes of rs-fMRI data collection, and then a DTI scan, all within one session.

Connectivity was determined by comparing simultaneous signals from an area identified as the BNST, with

identified regions in the rest of the brain. Though it is an important nucleus, the human BNST is less than 190mm^3, with boundaries that are difficult to identify. To create a template, or mask, to identify the BNST, Avery and colleagues collected an ultra-high-resolution 7-tesla image from one 42-year-old male. The detailed outline of the BNST from this precise 3-dimensional study was then placed onto the lower resolution images obtained from all the other subjects, using their visible anatomical boundaries. Differences in signal from the masked area were measured to determine structural and functional coincidence with 54 brain regions identified through the Harvard-Oxford Probabilistic Atlases, which did not contain mid-brain structures, such as the hypothalamus.

Images were analyzed using an agnostic approach to describe BNST relationships to target areas, both ipsilaterally and contralaterally. In addition, an innovative statistical approach was applied to the DTI data, evaluating the number of tractography streamlines passing from each seed voxel, allowing for a better estimate of long distance connectivity. Regions of significance, which were above the threshold value in more than 50% of the individual participants, were reported to have connectivity with the BNST, a stringent statistical paramater.

This conservative statistical approach revealed 17 regions with a significant likelihood of structural connectivity to the BNST, confirming previously known BNST relationships between the basal ganglia (accumbens, caudate, putamen, pallidum), amygdala, subcallosal cortex, hippocampus, and thalamus. Surprisingly, the BNST was also found to be connected to the temporal pole region, a paralimbic area, which is presumed to bind complex perceptual inputs to strong emotional responses. Functional connections to the basal ganglia (accumbens, caudate, putamen, pallidum), hippocampus, and thalamus were also confirmed, and a novel functional circuit was found between the BNST and the paracingulate gyrus, a part of the prefrontal cortex, which is thought to mediate responses to complex social pressures.

Structural and functional findings converged on connectivity between the BNST and the accumbens, thalamus, hippocampus, pallidum, caudate and putamen. Avery et al. propose that these regions form the nexus of the human BNST circuit. Surprisingly, the amygdala and subcallosal cortex showed structural, but not functional connectivity, though prior work had linked these regions strongly with the BNST. This may have been due to the conservative statistical approaches used, or it may reflect the limitations of fMRI to image increased GABAergic projections, rather than glutamatergic projections.

Within the amygdala, this study confirmed previously established connections between subnuclei. The strongest structural connection was between the BNST and the accumbens and it was the second strongest functional connection as well, a finding which is exciting because both structures feature prominently in anxiety and addiction neurocircuitry, but their relationship had not been previously confirmed.

Because the BNST is known to be gender dimorphic in rodents, sex differences were also explored, showing greater structural connectivity between the BNST and 76% of the connected regions in females. Functional connectivity between the left putamen and the BNST was greater in males, while females showed a stronger functional connection between the BNST and the right thalamus, pointing to an explanation for some of the gender differences in the prevalence of anxiety and addiction.

Hopefully, future studies will create a probabilistic atlas of the BNST combining multiple 7-tesla images for more accurate masking, and will explore BNST connectivity to the midbrain structures, which were not analyzed in this project. The BNST is a key nucleus for understanding chronic anxiety and addiction behavior, and future studies of these disorders will draw from these results to understand the underlying neural mechanisms.

This is a landmark study because it establishes, for the first time, the functional and structural neurocircuitry of the BNST in human brain, using a stringent and innovative statistical approach.

Suzanne N Avery, Jacqueline A Clauss, Winder, DG, Woodward, N, Heckers, S, & Blackford, JU (2014). BNST Neurocircuitry in humans, *Neuroimage* 91:311-23.

Turns out, you don't need both amygdalae to appreciate porn

A predominant theory of enhanced visual cortical processing of emotional stimuli is that the amygdala modulates the amount of attention given to visual stimuli and enhances the processing for stimuli that are emotionally arousing. Evidence for this has been put together from anatomical tracing studies in nonhuman primates between the amygdala and ipsilateral ventral visual stream areas, functional connectivity analyses of blood oxygenation level-dependent (BOLD) signals implicating connections of the amygdala with the visual stream, and patients with medial temporal lobe sclerosis showing reduced response to fearful faces than control subjects. However, behavioral results in patients with amygdala lesions suggest that there are other

brain structures that contribute to enhanced visual stream processing of emotional stimuli. In this study, Edmiston and McHugo et. al. examined the BOLD signals of patients with amygdala lesions in response to visual emotional scenes. They hypothesized that if the amygdala is responsible for the enhanced effect of visual stream processing seen in previous studies, then the visual stream BOLD signal ipsilateral to the amygdala lesion should be reduced in these patients. Conversely, if another network of structures is responsible for modulating visual stream processing of emotional stimuli, then the ventral visual stream BOLD signal ipsilateral to the amygdala lesion should not be reduced.

To test their hypothesis, the authors used functional magnetic resonance imaging (fMRI) to collect BOLD signals of control participants (n=16) and patients who had undergone unilateral temporal lobe resection that included removing part or all of the amygdala from either the right (n=13) or left (n=5) hemisphere. Most studies that explore visual stream processing in response to emotional stimuli use images of faces expressing specific emotions (eg, fear, anger) or of neutral objects, and the participants are asked to view the images while performing a task. One confound of this type of paradigm is that faces elicit activation of specific brain regions, while scenes recruit a broader network for neural processing. Furthermore, tasks require top-down attentional control that may confound stimulus-driven neural processing. Thus, the authors of this study used emotional scenes of aversive, erotic, neutral, or scrambled images, and the participants viewed the images passively while in the scanner.

Consistent with previous findings, the control participants showed increased visual cortical activation in response to the aversive and erotic images compared to the neutral images, specifically in bilateral primary and secondary visual cortices for both aversive and erotic conditions and additionally the fusiform gyrus for the aversive condition. For the amygdala-resection patients, bilateral primary and secondary visual cortices showed enhanced BOLD signal for aversive and erotic images compared to neutral images. Because the amygdala resections were unilateral, an increased BOLD signal of visual cortex contralateral to the lesion compared to that of visual cortex ipsilateral to the lesion would indicate that the intact amygdala still contributes to the enhanced cortical response. To investigate this possibility, the authors compared the ipsilateral and contralateral BOLD signals of the primary visual area, lateral visual association cortex, and the fusiform gyrus and found no significant differences in activation of these areas between the two hemispheres. Next, the authors examined if the size of lesion had an effect on cortical activity. This analysis revealed a positive correlation between lesion size and BOLD signal of the contralateral fusiform gyrus in the aversive condition and contralateral visual association cortex in the erotic condition. This suggests that increased lesion size might lead to compensatory activity of contralateral visual cortical processing.

The results of this study challenge the classic model that the amygdala modulates bottom-up visual cortical processing of emotional stimuli. Instead, it supports an alternative hypothesis that there are multiple parallel pathways that mediate the processing of salient, particularly aversive and erotic, stimuli. This study opens the door for future studies to examine what other structures may be involved in modulating visual stream processing and what other networks contribute to our processing of emotional scenes.

E. Kale Edmiston, Maureen McHugo, Dukic, MS, Smith, SD, Abou-Khalil, B, Eggers, E & Zald, DH (2013). Enhanced visual cortical activation for emotional stimuli is preserved in patients with unilateral amygdala resection. *J Neurosci* 33(27):11023-31.

RESEARCH BRIEFS

Serotonin System Regulation in Autism Spectrum Disorder: Functional Analysis of Rare Coding Variants of the Adenosine A3 Receptor

Autism Spectrum Disorder (ASD) is a complex neurodevelopmental disorder characterized by deficits in social behavior as well as by restricted and repetitive behaviors. Because of the heterogeneity within ASD, the genetic basis of the disorder is likely linked to interacting factors that moderate risk. Studies that screen for rare genetic variants in ASD are important because they can help to elucidate biological mechanisms that might underlie pathophysiology of the disorder or specific symptoms associated with multiple disorders. In a recent study by Vanderbilt Brain Institute graduate student Nicholas Campbell, the authors examined the functional impact of two rare variants of the gene encoding the A3 adenosine receptor (ADORA3) in a sample of families with one or more members diagnosed with an ASD. The ADORA3 receptor is expressed at the synaptic terminals of neurons that synthesize serotonin; variants of the ADORA3 gene have been linked to regulation and expression of the

serotonin transporter via the p38 MAPK and PKG signaling cascades. The authors found an increased number of rare coding variants in ADORA3 in cases compared to controls. The authors performed a functional analysis of two of these rare inherited variants- Leu90Val and Val171Ile. These functional studies suggested effects on serotonin transporter regulation in these two alleles- with the Leu90Val receptor variant associated with increased serotonin uptake and the Val171Ile receptor variant associated with a downstream decrease in serotonin transporter-mediated uptake. These findings suggest dysregulation of the serotonin system in ASD and implicate a functional mechanism that confers increased risk for development of ASD.

Nicholas G. Campbell, Zhu, CB, Lindler, KM, Yaspan, BL, Kistner-Griffin E, NIH ARRA Consortium, Hewlett, WA, Tate, CG, Blakely, RD, Sutcliffe, JS. (2013). Rare coding variants of the adenosine A3 receptor are increased in autism: on the trail of the serotonin transporter regulome. *Mol. Autism* 4(1):28.

Heavy Metal – Not Your Typical Concert: The Role of PD Related Genes in Metal Handling

Parkinson's disease (PD) affects 1% of the population, yet its underlying mechanisms remain elusive, and may be due to an unknown combination of both genetic and environmental factors, such as manganese (Mn) exposure. Known cellular manifestations of this debilitating neurodegenerative disease include aggregates of alpha-synuclein (a-Syn), mitochondrial dysfunction, and oxidative stress. Though the majority of PD cases are idiopathic, a very rare form of early-onset familial PD is due to a recessive gene mutation on one of a trio of genes, PARKIN, PINK1 and DJ1, which seem to be involved in diverse molecular pathways. A recent study from the Aschner lab investigated the role of these genes in controlling the cellular response to Mn exposure, using an invertebrate nematode model with a knock-in of human a-Syn, as well as single deletions of the relevant PD genes.

Mn exposure alone causes oxidative stress, and thus can reveal impairments in cellular stress response as well as alterations in metal handling. To investigate these pathways, *Caenorhabditis elegans* (*C. elegans*) were exposed to Mn and then analyzed for Mn content, survivability, and the presence of reactive oxygen and nitrogen species (RONS). Dopaminergic neuronal degeneration was also assayed, as it is a hallmark symptom of PD.

As is usually the case with PD research, the results were unexpected, but interesting. According to dose-response survival curves, worms with deletions in *pdr1* (homologous to PARKIN) were hypersensitive to Mn toxicity, while those with deletions of *djr1.1* (homologous to DJ1) were less sensitive than wild type. *C. elegans* does not express a-Syn naturally, but when the nematodes also carried the human a-Syn knock-in, they became more susceptible to Mn exposure. Both the *pdr1* and *djr1.1* mutants accumulated more Mn than wild type worms, a result that echoed prior studies in *Drosophila* showing *dj1* mutations to be related to increased metal accumulation. The worms that bore a-Syn demonstrated resistance to Mn accumulation, adding to evidence of a protective metal-binding function for a-Syn.

All of the deletion mutants (including *pink1*) had increases in baseline RONS, and decreases in baseline glutathione levels, revealing an impaired ability to respond to oxidative stress. Mn-exposure exacerbated RONS levels, but the presence of a-Syn reduced RONS in the *pdr1* and *djr1.1* mutants, further supporting a neuroprotective role for a-Syn. In wild type worms, degeneration of dopaminergic neurons was not increased by Mn exposure, although the presence of a-Syn served to protect them from normal degeneration in the presence of the *pdr1*, but not the *djr1.1* mutation.

The nematode model used in this study provides a tool to examine the interactions between genes known to cause PD, and an environmental exposure, which is also associated with similar neurodegeneration. Results point to a net of interactions between manganese and the known familial PD genes, implicating alterations in metal handling as a part of the root cause of PD. In addition, the presence of human a-synuclein provided a protective role against oxidative stress and Mn toxicity when genes known to cause familial PD were deleted, a finding which suggests that more research into the metal-binding capacity of a-Syn is warranted.

Bornhorst, J, **Sudipta Chakraborty**, Meyer, S, Lohren, H, Brinkhaus, SG, Knight, AL, Caldwell, KA, Caldwell, GA, Karst, U, Schwerdtle, T, Bowman, A & Aschner, M. (2014). The effects of *pdr1*, *djr1.1* and *pink1* loss in manganese-induced toxicity and the role of α-synuclein in *C. elegans*. *Metallomics* 6(3):476-90.

Basal metabolic differences in the human subiculum

The hippocampal formation is a brain structure involved in memory, spatial navigation, and mood, and abnormalities in this structure are associated with neurologi-

cal and psychiatric disorders such as Alzheimer's disease and schizophrenia. Multiple subfields make up the hippocampal formation, including the hippocampus proper (CA1-4), dentate gyrus, and subiculum. Previous work has shown that there are anterior-posterior gradients in the hippocampal formation with regards to cell number, neurochemistry, and metabolism; however, less is known about the differences in metabolism within the human hippocampal subfields. In this study the authors use contrast-enhanced steady state imaging to measure cerebral blood volume (CBV), a marker of basal metabolism, of the hippocampal formation and its subfields along the anterior-posterior axis in fourteen healthy human subjects. The authors identify significant CBV gradients in the left and right hippocampal formation. Upon further examination, the authors localize the CBV gradient to the left and right subiculum, with the highest CBV levels occurring in the anterior regions. This anterior to posterior CBV gradient suggests that the anterior subiculum is more active than the posterior subiculum. The subiculum is an important outflow subfield of the hippocampal formation, but the functional implications of this gradient are unclear. However, it is likely that disruptions in these natural gradients within the hippocampal formation will advance our understanding of complex neuropsychiatric disorders, such as schizophrenia.

Pratik Talati, Rane S, Kose S, Gore J, Heckers S. (2014). Anterior-Posterior Cerebral Blood Volume Gradient in Human Subiculum. *Hippocampus.* 24:503-509.

Amygdala and Basal Ganglia Structure and Function in Adults with Inhibited Temperament

Inhibited temperament (IT) is a hereditary, stable, and biologically-based trait associated with increased risk for a host of psychiatric disorders. IT individuals are avoidant of novel stimuli and situations, timid, and slow to approach new people. Jacqueline Clauss, a graduate student in the Blackford Lab, conducted a magnetic resonance imaging (MRI) study of the neuroanatomical and functional correlates of IT, recently published in *Social Cognitive and Affective Neuroscience*.

A sample of young adults with IT was compared to those with extreme uninhibited temperament. Because prior research has implicated the amygdala in novelty detection, the authors were particularly interested in shape and volume differences in the amygdala between groups. The authors found that IT individuals had overall larger amygdala volumes than uninhibited participants, with greater volumetric differences in the basal and lateral subnuclei. Shape analysis also showed increased convexity in these same amygdala subregions. To determine the relationship between amygdala network connectivity and volume, the authors performed functional connectivity analyses on a subset of participants who also underwent functional MRI (fMRI) using a novel and familiar face viewing paradigm. Increased volume of the amygdala was significantly correlated with greater connectivity between the amygdala and the opposing temporal lobe during face processing. Greater right amygdala volume was also associated with increased functional connectivity in the visual cortex, including the fusiform gyrus, and the insula. These correlation analyses reveal a relationship between greater amygdala volume and a network of brain regions associated with social and emotional stimulus processing.

Jacqueline A Clauss, Seay, AL, Vanderklok, RM, Avery, SN, Cao, A, Cowan, RL, Benningfield, MM & Blackford, JU (2014). Structural and functional bases of inhibited temperament. *Soc. Cogn. Affect. Neurosci.* 10.1093/scan/nsu019.

On the Cover

Each year we ask the qualifying class to submit images for the VRN cover art competition. This year's winner is Britney Lizama-Manibusan . Here she describes her project and how it relates to the cover image, as well as some information about herself.

"Stroke is the 4th leading cause of death and the leading cause of long-term disability in the United States. Given that over 80% of strokes are ischemic in nature, there is a particularly urgent need for pre-clinical investigation of the pathophysiology of ischemic stress to identify processes that enhance cell survival in response to injury. Interestingly, the brain has powerful means to adapt to acute ischemic stress through an endogenous form of protection called preconditioning (PC). We have developed both *in vivo* and *in vitro* models of PC to understand the molecular underpinnings of endogenous protection. In our *in vitro* model, primary rat cortical neurons are treated with mild oxygen and glucose deprivation and allowed to recover in growth media. During the recovery period, we can monitor the protein profile of these neurons through immunoblotting and immunocytochemistry techniques. The cover photo depicts preconditioned neurons stained with neuronal marker MAP2 (green), nuclei marker DAPI (blue), and the molecular chaperone Heat Shock Protein 70 (HSP70, magenta). HSP70 is increased in preconditioned cultures and is essential to neuroprotection afforded by PC. However, while HSP70 is essential, it is not sufficient to promote cell survival. My thesis project focuses on identifying other proteins important in determining cell fate after PC in an effort to find biomarkers of cell survival and targets for therapy after CNS injury.

I graduated from the University of Arizona with a Bachelor of Science degree in Molecular and Cellular Biology in 2011. I am currently a fourth year student in the Neuroscience Graduate Program doing my thesis work in Dr. BethAnn McLaughlin's lab."

Britney N. Lizama-Manibusan

Ocular Blast Trauma: Models, Mechanisms, and a Potential Therapeutic Strategy

Courtney M. Bricker-Anthony

Ocular blast trauma is a comorbid condition with traumatic brain injury and affects 45% of veterans from recent conflicts. Closed globe injuries impacting the retina can remain initially undetected in blast-exposed veterans and result in vision loss over time. The cellular and molecular mechanisms promoting retinal cell death and visual dysfunction are currently unknown, and no therapeutic agents are available for the treatment of this injury. This review will discuss current animal models of ocular blast trauma, potential mechanisms of retinal cell death and visual dysfunction, and the efficacy of erythropoietin, an endogenous neuroprotective cytokine, as a treatment.

Keywords *Blast injury, closed globe trauma, vision loss, cell death, erythropoietin*

The signature wound of the recent conflicts in Iraq and Afghanistan is blast injury[1], which impacts both military and civilian populations. Blast exposure has numerous negative ramifications on the human body, including damage to the lungs and central nervous system[2,3]. Traumatic brain injury (TBI) from blast exposure is heavily studied[4-6]. However, ocular blast trauma (OBT), a frequent comorbidity with TBI[7,8], is relatively understudied. Blast exposure can cause open or closed globe injuries. Closed globe injuries include superficial lacerations and contusions to the globe from a blunt force or overpressure wave[9]. Open globe injuries include penetrating or perforating wounds to the cornea or sclera[9]. Post-trauma vision is poorer in patients with open globe injuries[10], but closed globe injuries can remain undetected until appreciable vision loss occurs[11].

This review will discuss: 1) the key findings of three *in vivo* models of OBT in mice and rats[12-14], 2) the potential mechanisms underlying visual dysfunction and cell loss in each model, and 3) the therapeutic potential of erythropoietin for the treatment of OBT.

Current OBT Models

Modified Paintball Gun: The Contributions of Anterior Chamber Damage and Changes in Intraocular Pressure to the Pathogenesis of OBT

Our lab introduced an OBT model that isolated the impact of an overpressure blast wave to the mouse eye alone[12]. Some of the more interesting initial findings from our model system include decreases in intraocular pressure (IOP). A decrease in intraocular pressure following OBT indicates possible damage to the cornea, ciliary body (the structure responsible for production of aqueous humor), or trabecular meshwork (a network of collagen fibers through which aqueous humor exits the anterior chamber). If the trabecular meshwork is torn, this could increase outflow of aqueous humor through Schlemm's canal and cause a decrease in IOP. Scarring of an injury in the trabecular meshwork could eventually lead to a decrease in aqueous outflow, leading to the development of traumatic glaucoma. Also, damage to the ciliary body could disrupt production of aqueous humor and lower IOP. Another possible explanation is inflammation within the anterior chamber, which can increase blood-aqueous permeability and lower production of aqueous humor in the ciliary body[15]. Damage to the anterior chamber is associated with development of traumatic glaucoma[16,17]. To investigate the possible development of traumatic glaucoma, we would need to image the anterior chamber using optical coherence tomography (OCT)[a] and continue monitoring IOP over time to detect any abnormal increases. Additionally, if increased IOP is detected, we should also test retinal ganglion cell function using visually evoked potentials.

Our model put forth new avenues of inquiry to further understanding the pathogenesis of OBT. However, there are some questions about our experimental construct. Blast exposure causes simultaneous damage to the eye and the brain. Isolating exposure to the eye is not a true representation of the impact of OBT on the entire visual system. While this isolation poses a potential disadvantage, it is also advantageous because we demonstrate that an overpressure blast wave directly affects the retina, and we can use this approach to investigate the mechanisms of blast injury. In future experiments, we need to compare the effects of isolated

a. Optical coherence tomography is an *in vivo* imaging technique that utilizes near-infrared light and a special camera to collect images of the retina, optic nerve head and anterior chamber. It is used in both animal studies and in ophthalmic clinics.

versus whole head blast. An additional area of concern is gender. All the mice used in our initial experiments were female. Estrogen is protective in experimental head trauma and may also protect pre-menopausal women from cataracts and ocular disease[18,19]. We need to study the responses of male mice to OBT and determine if any sex differences exist.

Blast Chamber: The Impact of Inner Retinal Pathology on Visual Outcomes

In contrast with our model, Mohan and colleagues adopted a whole head exposure strategy and characterized their model system in adult male mice[13]. Their model characterization yielded many fascinating results. Some intriguing findings included their pattern ERG (pERG)[b] data and immunohistochemistry.

The pERG results from Mohan and colleagues suggest substantial dysfunction or loss of retinal ganglion cells following OBT. However, the researchers only collected pERG data at acute time points and one year post-injury. Longitudinal assessment of the pERG response would reveal the development of deficits over time and validate its use as a potential biomarker for the diagnosis of OBT pathogenesis.

Immunohistochemistry is a good first step for identifying potential markers of pathology, such as 4-HNE, iNOS and beta amyloid, in tissue sections. However, attempting to quantify these markers with relative expression scores is inadequate. If the authors wished to truly quantify these markers, then they should have performed western blots or ELISAs to confirm increases in protein levels. The immunohistochemistry results would have been useful for showing localization of the markers to suspected regions of damage, i.e. the ganglion cell layer. We have also examined retinal sections for the presence of 4-HNE and 3-nitrotyrosine (a product of iNOS) three days post-injury. We saw increases in 3-nitrotyrosine labeling, but no changes in 4-HNE. However, a recent rat model of TBI showed increases in 4-HNE and 3-nitrotyrosine at 3 hours post-injury that returned to baseline levels by 24 hours[20]. These findings indicate an early, shared mode of pathogenesis (oxidative stress) in both traumatic brain injury and OBT.

Explosives: Implications of Photoreceptor Cell Loss and Blood-Retinal Barrier Permeability

Zou and colleagues documented photoreceptor cell death, glial reactivity and inflammation in adult male rats following OBT[14]. In contrast with the findings of Mohan and others, the most heavily impacted cell population in this OBT model appeared to be the photoreceptor cells. Loss of photoreceptors occurs in retinitis pigmentosa, age-related macular degeneration, and diabetic retinopathy. Substantial photoreceptor cell death is devastating to retinal function, especially in areas of high acuity vision like the fovea. However, without functional assessments, it is difficult to discern whether or not the photoreceptor cell loss reported in this study was significant enough to result in visual deficits.

The authors noted extensive Müller glia reactivity following OBT. Increased expression of glial fibrillary acidic protein (GFAP) within Müller glia is a hallmark of retinal pathology and appears in retinal degeneration, injury, glaucoma, and diabetic retinopathy[21-23]. GFAP also increases in astrocytes following experimental TBI and is gaining popularity as a serum biomarker of TBI[24,25].

Glial reactivity in both OBT and TBI continues a trend of common findings between the two tissues. However, the aquaporin-4 expression in this OBT model seems to disagree with data from TBI. For example, aquaporin-4 expression decreases following experimental TBI and is associated with reduced water transport and promotion of cerebral edema[26-28]. In ischemic retinal injury, a complete knockout of aquaporin-4 in mice was shown to protect against retinal edema, inner retinal cell loss and flash ERG[c] deficits[29]. Aquaporin-4 might play different roles in TBI versus OBT. If this pattern holds true in future studies, any treatment strategy attempting to reduce retinal edema by targeting aquaporin-4 will need to be tissue-specific to prevent damaging off-target effects in the brain.

Cell Death In OBT

Cell death is a common consequence of ocular disease and injury and occurs in all three models of OBT[12-14]. As previously discussed, oxidative stress, inflammation, and reactive gliosis are all possible contributors to OBT pathogenesis. Each of these stressors can potentially trigger either apoptosis (caspase-dependent cell death) or necroptosis (caspase-independent cell death). Our lab has studied cell death following OBT, and we have noted paucity in caspase-3 positive nuclei in areas of cell death marked by

b. Pattern electroretinogram is a non-invasive test of retinal ganglion cell function. The test involves placement of a ring electrode on the surface of the cornea and measuring the electrical responses of the eye to reversals in a monochrome checkerboard pattern on a monitor.

c. The flash ERG is similar to the pattern ERG in that both tests use a corneal electrode to measure electrical responses. However, in the flash ERG, the stimulus consists of flashes of white light at different intensities that preferentially excite photoreceptor cells and ON bipolar cells.

TUNEL (an *in situ* hybridization assay that detects nicked DNA in dead and dying cells) at multiple time points post-injury. We hypothesize that necroptosis contributes to cell death following OBT. Thus, the mechanisms of necroptosis in retinal injury will be reviewed.

TNF-α, a pro-inflammatory cytokine upregulated following OBT injury, is capable of inducing both apoptosis and necroptosis[14,30]. Once TNF-α binds TNFR1, it can cause one of three outcomes. TNF-α can promote cell survival via NF-κB activation[31]. It can also lead to formation of either the apoptosome, which drives caspase-dependent cell death, or the necrosome, which drives caspase-independent cell death[30,32]. Whether a cell commits to apoptosis or necroptosis appears to be dependent upon the ubiquitination status of RIP1 and activation of caspase-8[33,30]. When RIP1 is deubiquitinated, it can form the apoptosome with active caspase-8 or the necrosome with RIP3 when caspase-8 is inhibited[30].

RIP3 is necessary for execution of necroptosis and has been shown to cause excess production of reactive oxygen species (ROS) in mitochondria, a potential mechanism of necrotic death[32]. Huang and colleagues reported expression of RIP3 in the inner retina of control rats[34]. Our lab has also documented inner retinal expression of RIP3 in the normal mouse retina. Upregulation of RIP3 was recently reported in a retinal injury model and may also play a role in OBT pathogenesis[34].

The data contributed by Huang and colleagues in their retinal injury model (acute elevation of IOP) raises many questions about the role of RIP3 in the normal and injured retina[34]. First, why is RIP3 expressed in nearly half of retinal ganglion cells and horizontal cells in normal conditions? Results from a series of *in vitro* experiments by Zhang and others show that RIP3 directly interacts with and increases the activity of several key enzymes involved in energy metabolism and that these interactions can be encouraged by application of TNF-α[32]. Basal levels of RIP3 protein within normal retinal neurons may regulate energy metabolism in a non-pathogenic manner. The high basal levels of RIP3 in inner retinal neurons may also make them vulnerable to necroptosis in response to certain stressors. In contrast, the photoreceptors of the outer retina appear to express very little RIP3 under normal conditions and in the context of retinal ischemia. This staining is consistent with mRNA expression patterns of RIP3 in the normal mouse retina reported by Trichonas and others[35].

Another important question pertains to the absence of RIP3 expression in propidium iodide positive inner retinal cells. While RIP1 expression is dispensable in some cases of necroptosis, RIP3 is required[30]. Given the loss of cell membrane integrity in those cells, it is possible that the protein was degraded amidst the cellular milieu. This possibility, as well as the colocalization of RIP3 with clearly apoptotic cells, drives home the importance of using secondary markers like propidium iodide or morphological characteristics (swollen cellular components lacking an intact cellular membrane, visible by electron microscopy) to confirm necroptotic cell death.

These studies demonstrate that both the inner and the outer retina are susceptible to necroptosis in certain pathological conditions. Necroptosis could also potentially contribute to cell loss in OBT, given the increases in oxidative stress reported in each model. Any potential therapeutic agent will need to protect retinal cells from both apoptosis and necroptosis to provide complete protection from OBT pathogenesis.

Erythropoietin: a Potential Neuroprotective Treatment for OBT

Structure, Function and Receptors

Erythropoietin (EPO)[d] is an endogenous cytokine best known for its role in stimulating hematopoiesis and is clinically approved for the treatment of anemia[36]. There are two binding sites for the EPO receptor homodimer on the surface of the protein, consisting of high affinity and low affinity binding sites[37]. EPO mRNA is heavily expressed in the kidney and liver, but it is also present in neuronal tissue[38]. Many laboratories became interested in EPO when it was shown to exert neuroprotection *in vitro*[39]. However, systemic treatment with wild-type EPO can lead to the development of polycythemia, a potentially life-threatening condition.

To combat the unwanted erythropoeitic "side effect" of wild-type EPO when used for neuroprotection, Leist and colleagues generated several modified forms of EPO[40]. Carbamylation of EPO (CEPO) resulted in a dearth of binding to the EPOR homodimer, yet it remained neuroprotective both *in vitro* and *in vivo*[40]. Two separate mutations in the low affinity binding site, S100E and R103E, were also effective at preventing binding to the EPOR homodimer while maintaining neuroprotection[40]. Our lab also developed a mutant form of EPO, EPO-R76E, which protected retinal ganglion cells in DBA/2J glaucomatous mice

d. EPO is a cytokine primarily produced in the kidney. It stimulates red blood cell production upon binding to the EPOR homodimer. A version of the EPOR is also expressed in neural tissue, including the retina, and exerts neuroprotection upon binding of EPO.

and displayed attenuated erythropoiesis, indicative of poor binding to EPOR homodimer[41]. Brines and colleagues later demonstrated that EPO and CEPO bind an EPOR and ILβ-subunit R heterodimer, which provides tissue protection without stimulation of hematopoiesis[42].

Protective Mechanisms

Though EPO is neuroprotective in multiple models of neuronal stress and disease, its precise mechanisms are still unclear. The addition of neuroprotective EPO mutants with attenuated or abolished hematopoietic activity by Leist and colleagues and our lab also complicates the question of EPO's protective mechanisms[40,41]. Binding of EPO to its native receptor homodimer initiates erythropoiesis via the Jak/Stat signaling cascade and appears to promote erythrocyte survival via GATA-1-mediated upregulation of anti-apoptotic Bcl-XL[43,44]. However, EPO's neuroprotective pathways appear to vary even among neuronal tissues.

In an *in vitro* model of neuronal hypoxia, administration of EPO induced increased phosphorylation of Stat5, Akt, ERK1 and ERK2 in hippocampal neurons[45]. Addition of inhibitors of the MAPK and PI3K pathways in conjunction with EPO treatment prevented phosphorylation of ERK1, ERK2 and Akt and abolished EPO neuroprotection[45]. Activation of the MAPK pathway provides protection via inhibition of pro-apoptotic BAD and phosphorylation of CREB, which transcribes pro-survival genes[46]. Phosphorylation of Akt within the P13K pathway also prevents activation of BAD and leads to downstream activation of Nf-κB, which promotes transcription of pro-survival genes[47]. Another *in vitro* model of excitotoxicity in cerebrocortical neurons showed that Jak2-mediated activation of Nf-κB was necessary for EPO neuroprotection[48]. Together, these findings support a common pathway for EPO neuroprotection, as Jak2 contributes to both MAPK and PI3K signaling cascades[49,50].

However, findings from Weishaupt and colleagues challenged the notion that MAPK signaling was involved in EPO neuroprotection within retinal ganglion cells (RGCs)[51]. To test the efficacy of EPO neuroprotection in RGCs, the authors used both an *in vitro* (trophic factor deprivation in RGC cultures) and an *in vivo* (optic nerve transection, an acute model of glaucoma) approach. They detected EPOR on the RGCs of both control and optic nerve transected rats and thus claimed that the EPOR expression was much weaker in other cell types of the retina. However, this finding disagrees with data from Xie and others, who reported EPOR expression throughout the layers of the retina without strong localization within the RGCs in normal Sprague-Dawley rats, the same rat breed used in the Weishaupt study[52]. Grimm and colleagues also reported a different EPOR expression pattern within the normal mouse retina (strong staining within photoreceptor inner segments and the outer plexiform layer, weak labeling within the inner retina), but these results could be due to a species difference[53]. In examining the results of these studies, it is clear that a current challenge in the field is the inconsistency of EPOR antibodies[54].

Surprisingly, EPO treatment elicited phosphorylation of Akt, but not of ERK1/2 in optic nerve transected retinas. Both the MAPK and PI3K pathways are active and involved in EPO neuroprotection in cerebral ischemia and intracerebral hemorrhage[45,55,56]. A possible explanation for the lack of ERK1/2 phosphorylation in this study is that the neuroprotective cascade initiated by EPO differs in RGCs compared to cerebral neurons. However, a recent study demonstrated that inhibition of MAPK, PI3K, or Stat5 in cultured rat RGCs challenged with trophic factor withdrawal, TNF-α, or NMDA resulted in a significant loss of EPO neuroprotection[57]. However, the reduction in EPO neuroprotection in the aforementioned study varied among RGC types and the type of insult. These findings suggest that different cytotoxic stimuli and cell types may result in alterations in EPO's neuroprotective signaling cascades, which needs to be explored further in future experiments.

Conclusions

OBT is a complex injury with many possible modes of pathogenesis. In patients, damage appears to occur in both the anterior and posterior poles, which has serious consequences for regulation of IOP, immune privilege, and visual function. The current models of OBT have highlighted several areas that may drive injury pathogenesis, including loss of immune privilege, axonal injury, oxidative stress, inflammation, and reactive gliosis. However, there will be challenges moving forward with OBT research. The current OBT model systems may represent different facets of OBT injury. Future directions for OBT research include bolstering our understanding of both the acute and long-term effects of OBT, teasing out the mechanisms underlying cell loss and visual dysfunction and testing protective therapeutics like EPO to see if OBT pathogenesis can be circumvented with treatment.

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Regulation of AMPA-type Glutamate Receptors in LTP and Acute Stress

Victoria S. Cavener

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors are tetramers of GluA1-4 subunits that mediate normal excitatory transmission. Multiple forms of synaptic plasticity, such as long term potentiation (LTP), are caused by changes in the activity, number or surface expression of synaptic AMPA receptors (AMPARs). Homo-tetrameric GluA1 AMPARs are unique in that they form calcium-permeable AMPARs (CP-AMPARs), which can initiate downstream signaling without the membrane depolarization that is required for calcium influx via voltage-gated calcium channels or NMDA-type glutamate receptors. It is well established that multiple scaffolding and signaling proteins, as well as adrenergic receptors, associate with and modulate CP-AMPARs. Recent studies have shown that regulation of these supramolecular CP-AMPAR complexes in subcellular microdomains may play a key role in synaptic remodeling of the limbic system following episodes of acute stress. Thus, dysregulation of CP-AMPARs is emerging as a key element of the mechanisms underlying stress-induced anxiety disorders.

Keywords *CP-AMPARs, SAP97, CaMKII, stress signaling, adrenergic receptors, GluA1*

Environmental stress incites the limbic system to coordinate emotional experiences such as fear, reward, and motivation, with episodic memory to facilitate behavior adaptations. The molecular adaptations required for behavioral flexibility are unique to the particular function and precise variety of the neurons within each limbic region. This, coupled with experimental variables such as the type, duration, and strength of the environmental stressor, make it difficult to build a cohesive model explaining how the effects of acute and chronic stress affect limbic signaling[1-5]. Each layer of synaptic regulation provides the organism's brain with another potential tool to modify behavior in order to gain efficiency and resilience in a dynamic environment. As a consequence of this adaptability, organisms are vulnerable to disruption at any tier of plasticity that interrupts the balance of limbic signaling[6]. Chronic stress induced illnesses can be traced to disrupted neuronal plasticity at the molecular level[2]. The goal of this review is to briefly discuss how particular receptor/protein complexes may function to sensitize synapses in response to stress to facilitate learning. In order to propose this model, it is necessary to first discuss normal α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor (AMPAR) expression and trafficking mechanisms essential for synaptic plasticity. Following a brief explanation of the acute stress response, key proteins involved in long-term potentiation (LTP) are discussed in context of a proposed model where protein-protein interactions control perisynaptic microdomains to regulate the acute stress response.

AMPARs Are Essential Regulators of Synaptic Plasticity

Neurons communicate through a complex network of excitatory, inhibitory and modulatory chemical signaling mechanisms. Excitatory signals are primarily driven by glutamate release, and are received by tightly regulated glutamate receptors packed into and around the postsynaptic density (PSD) of glutamatergic synapses. The ionotrophic glutamatergic receptors include N-methyl-D-aspartate receptors (NMDARs), AMPARs, and kainate receptors. The signaling interface formed by the PSD controls important second messenger cascades and membrane potential changes necessary to generate an action potential. By these mechanisms, the combined synaptic activities of glutamate receptors drive long-term potentiation (LTP), de-potentiation and/or long term depression (LTD) of synaptic strength and are thought to be the essential mechanisms underlying learning and memory (Figure 1). Synaptic development, efficacy, and strength are heavily dependent on the expression of AMPARs and are sensitive to changes due to various combinations of AMPAR subunits contained in a single receptor[7]. The number and distribution of AMPARs at the synapse and their subsequent incorporation into the PSD is a direct molecular substrate for LTP. Conversely, when AMPARs are removed from the potentiated synapse, the signal transmission either de-potentiates to baseline or is depressed from baseline to subdue synaptic transmission (Figure 1).

AMPARs are hetero- or homo-tetramer ion channels comprised of a combination of four different subunits that confer different conductance properties to the ion channel. AMPARs including only GluA1 subunits are primarily present in immature/naïve synapses. However, newer evidence is gathering that calcium-permeable AMPARs (CP-AMPARS) are also important in the plasticity of mature synapses[8] (Figure 1). The lack of GluA2 subunits allows for the AMPAR to be CP-AMPARs. The GluA2

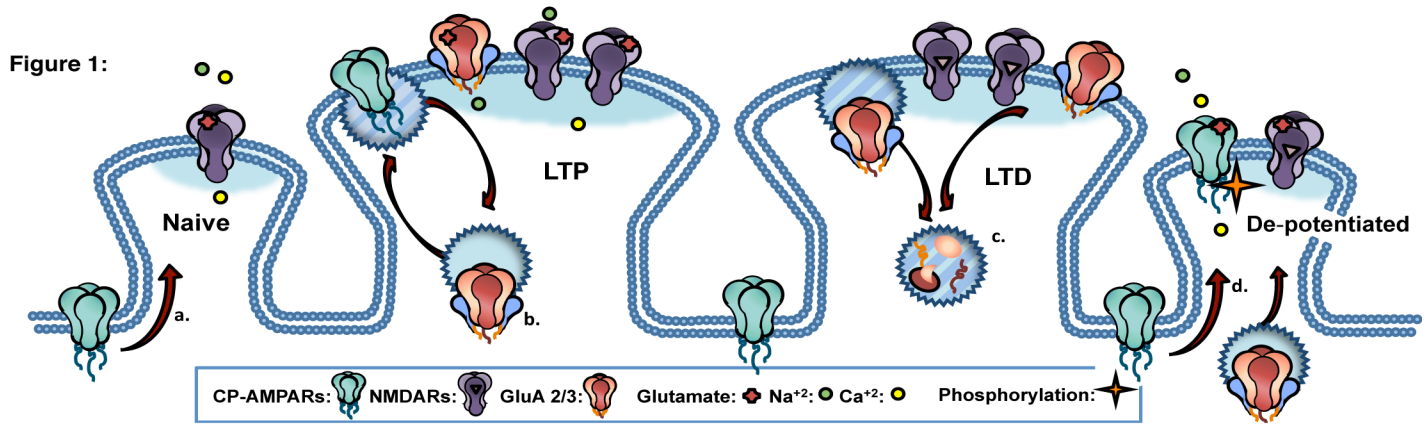

Figure 1. *Synaptic plasticity requires rapid trafficking of AMPAR in and out of the membrane.* a.) Glutamate signaling causes CP-AMPARs to diffuse into the peri-synaptic space. b.) After induction of LTP, CP-AMPARs are replaced by GluA2/3 AMPARs to stabilize the active synapse. c.) LTD occurs during low frequency stimulation to weaken the synapse by removing ion channels from the synapse to be recycled or degraded. d.) When a synapse is de-potentiated it has returned to baseline activity or is silenced. If CP-AMPARs have not been de-phosphorylated by PP2B at Ser845, and have been phosphorylated by CaMKII at Ser831, it can remain in the synapse. Future activation recruits more CP-AMPARs. This allows low glutamate signaling to cause calcium influx that triggers LTP at lower membrane potentials.

subunit prevents calcium (Ca^{2+}) influx through the receptor's ion channel due to an RNA editing event that changes an uncharged glutamine amino acid to a positively charged arginine (Gln-Arg) in the second trans-membrane domain of the ion pore[9] (Figure 2). AMPAR subunit changes were thought to stabilize synaptic transmission as the organism matures, but recent data show that GluA1 subunits are upregulated when there is an abnormally high burst of synaptic activity in mature animals[10]. After the initiation of LTP, GluA2/3 AMPARS and GluA1/2 replace the GluA1 receptors in the synapse. This molecular switch stabilizes the synapse at basal levels of excitatory transmission[11] (Figure 1b). This has led to speculation that CP-AMPARs serve as a voltage-independent source for Ca^{2+} signaling essential to initiate synaptic remodeling required for LTP in mature synapses[12].

Both the AMPAR subunits and accessory proteins are subject to post-translational modifications that regulate the expression and mobility of the receptor. Activated Threonine or Serine kinases work in tandem or in opposition of one another to facilitate signaling cascades that arbitrate AMPAR traffic, receptor kinetics, and the stability of the AMPARs after insertion into the synaptic membrane. Phosphorylation events mediate the interactions between auxiliary subunits and AMPARs. Kinase activity in the synapse impacts synaptic potentiation through receptor binding but also is important for genetic expression, protein synthesis, as well as the fate and maintenance of individual synapses.

The reversible phosphorylation of the AMPAR GluR1 subunit on the C-terminus tail is a post-synaptic mechanism known to regulate both AMPAR trafficking and channel conductance at the synapse (Figure 2). For a more detailed review of AMPAR trafficking see Shepherd and Huganir 2007[13]. Phosphorylation of GluA1 by calcium/calmodulin-dependent protein kinase II (CaMKII) at serine 831[14] and protein kinase A (PKA) at serine 845 potentiate AMPAR efficiency and enhance NMDAR-dependent LTP[15-16]. It is likely that phosphorylation provides fine control of synaptic strength by individual AMPARs and is dependent on particular accessory proteins present in peri-synaptic micro-domains[17-20]. When there is low cytosolic calcium, dephosphorylation of S845 by Protein Phosphatase 2B (PP2B-Calcinurin) is essential for endocytosis of the AMPAR and LTD[21-22]. Finally, protein kinase C (PKC) phosphorylation at Ser818 facilitates AMPAR exocytosis to the membrane[23]. In addition, a recent study provided evidence that phosphorylation of the GluA1 subunit by CaMKII at Ser831 serves to lower the threshold for LTP after fear learning and is required for fear renewal after fear extinction[24]. Figure 2 contrasts the phosphorylation sites of CP-AMPARS (GluA1 homomers) with non-Ca^{2+} permeable GluA2/3 AMPARs. For a more detailed review of how kinase activity regulates synaptic plasticity see Anggono and Huganir 2011[25].

Hormonal Signaling During Acute Stress Stimuli

Stress-induced activation of the hypothalamic-pituitary-adrenal axis (HPA axis) regulates several of the hormonal signaling cascades which directly affect synaptic plasticity. One of the first consequences of HPA axis activation is immediate release of norepinephrine (NE) from both the adrenal medulla and the locus coeruleus. NE binds to and activates a family of G-coupled protein receptors to inhibit or enhance cAMP signaling. β-adrenergic receptors are widely distributed in the brain and generally increase activity in cAMP second messenger cascades. Lesion studies in rodents that affect the NE-amygdala circuit show inhibited fear and appetitive learning. Anxiety disorders that involve persistent rumination over unlikely but highly anticipated stressors may be as a consequence of deregulation of the direct connections be-

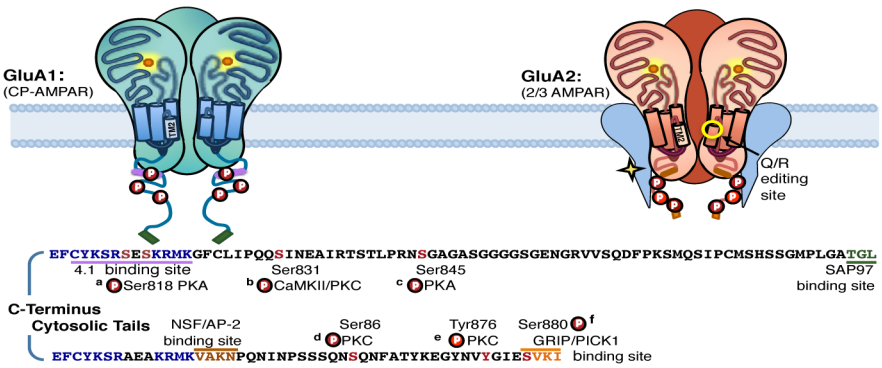

Figure 2. *Phosphorylation sites on the AMPAR subunits mediate subunit binding efficiency with auxiliary subunits, receptor surface expression and influence open channel probability.* These phosphorylation targets are located on the AMPAR subunit-specific c-terminal tail along with protein binding sites.

tween the locus coeruleus, cingulate gyrus, and amygdala.

Stimulation of the HPA axis causes secondary hormonal signaling with a longer and more enduring effect. Cortisol normally impacts alertness in the daytime in a rhythmic pattern opposite of melatonin to regulate circadian rhythm. Increased cortisol release triggered by the activation of the HPA axis causes cortisol to bind to mineralocorticoid receptors in the presynaptic membrane and promotes increased neurotransmitter release from the presynaptic terminal of effected synapses. When hippocampal slices were treated with cortisol, neurons had an enhanced response to NE signaling and AMPAR surface expression[26].

The remaining sections in this review focus on emerging evidence for protein-protein interactions that form supramolecular complexes with both CP-AMPARs and β2-Adrenergic receptors. These complexes may mediate synaptic responses after the sympathetic nervous system triggers the HPA axis during acute stress. Cortisol enhanced glutamatergic activity combined with norepinephrine signaling, stimulates activation of multiple signaling cascades that converge to prime the synapse for LTP[26]. These receptors are found in complexes in perisynaptic spaces of amygdala and hippocampal synapses. The discovery of these micro domains adds insight into particular mechanisms involved with fear extinction and anxiety.

Key Proteins of a Supramolecular Complex Are Important for Synaptic Plasticity

Each of the proteins implicated in this micro domain model for meta-plasticity are important for normal expression of LTP. Normal expression of these proteins has been studied extensively in relation to synaptic plasticity. In particular, proteins in this model affect AMPAR trafficking, LTP, and/or synaptic growth in a variety of ways. CaMKII is key in the regulation of LTP. For a detailed review about CaMKII regulation of synaptic plasticity see Lisman et al. 2012[27]. There is evidence to support that activated CaMKII is important in many if not all levels of AMPAR trafficking. It is the most abundant protein in the brain, and when its kinase activity is inhibited, LTP is completely blocked. In brain-specific CaMKII-inhibited rodent models, animals showed an inhibited acquisition of fear memory as well as spatial learning deficits. Additionally, when the CaMKII inhibitor is added to mouse brain slices, consolidation of memory in the prefrontal cortex (PFC) was repressed in a dose-dependent manner[28].

In the context of this review, CaMKII is the kinase responsible for two specific phosphorylation events. The first is pictured in figure 2. Phosphorylation of the GluR1 subunit at Ser831 is known to increase the open channel probability of the AMPAR[14]. Also, a recent discovery showed that removal of AMPARS with phosphorylated Ser831 was required for extinction of fear memory in the lateral amygdala. The study also showed that phosphorylation of Ser831 lowered the threshold for LTP in active synapses[24]. The second important CaMKII activity is the phosphorylation of a membrane-associated guanylate kinase (MAGUK) family protein called Synapse-associated protein 97 (SAP97)[29]. SAP97 is a scaffolding protein that regulates GluA1 via a multi-protein complex[18]. The PDZ2 domain of SAP97 preferentially binds with GluA1 on its last four c-terminus amino acids. SAP97 is the only MAGUK family protein able to bind GluA1 directly[18]. The alternatively spliced SAP97 U5 domain is thought to regulate SAP97 localization and also plays a role in regulating AMPAR trafficking and insertion to the membrane[30]. When the interaction between SAP97 and GluR1 is blocked, dendritic growth and branching is truncated[31]. One splice variant of SAP97's U5 region contains an I3 insert that was shown to bind CaMKII. When SAP97's I3 insert is bound and phosphorylated by CaMKII *in vitro*, SAP97 binding to AKAP 79/150 was interrupted[28].

A-kinase anchor protein AKAP79/150 is an anchoring protein for PKA, PKC, and calcineurin (PP2B). Particularly abundant in the PFC, it is primarily known to hold PKA in proximity of target proteins in the synapse. Localization of this protein complex is essential for cAMP signaling through PKA. PKA in association with this complex regulates the activation of beta-adrenergic receptors as well as the phosphorylation of Ser845 of the GluA1 subunit. This important complex also con-

tains calcium-activated calcineurin (PP2B). In this model, the phosphatase dephosphorylates Ser845. When GluA1-Ser845 dephosphorylation is prevented, LTD is blocked and AMPAR endocytosis is diminished[14]. These proteins function in a supra molecular complex in concert to promote calcium and cAMP signaling without NMDA activation. Prior activity in synapses enables these complexes to maintain lower thresholds for LTP. This may be a molecular mechanism for fear renewal and new memory formation.

Supramolecular Complexes Regulate Threshold for LTP in Response to Acute Stress

Recent research revealed a complex that includes a beta-2-adrenergic receptor (β_2AR), homomeric GluA1 AMAPAR, PKA, and PP2B. This assembly is dependent on the binding capabilities of SAP97[32]. A study that shows how CaMKII may interact with this complex has yet to be done. Emerging evidence of such interactomes offers an attractive model to explain how acute stress initiates rapid micro-domain signaling and lowers the NMDA-dependent synaptic threshold for LTP[28, 32]. Noradrenergic signaling through adrenergic GPCRs may regulate acute stress responses by forming a supramolecular complex with CP-AMPARs via SAP97. This complex may be an acute stress response to enhance plasticity by increasing CP-AMPAR surface expression and potentiate receptor channel activity at the same time. β_2AR forms a complex with βSAP97, AKAP150, CP-AMPAR, in a micro-domain that increases PKA phosphorylation of the GluA1 subunit at S845 and facilitates the PKC driven Ser818 CP-AMPAR insertion into the membrane[32-36]. This insertion increases calcium influx into the synapse and effectively promotes more AMPAR recruitment to the membrane. CaMKII activated by Ca^{+2} influx primes the synapse for the molecular restructuring required for LTP[32-36]. In addition, if CaMKII is activated and then bound by the SAP97 I3 insert within this micro domain, it will not only disrupt AKAP's association with SAP97-I3 but is in an ideal position to potentiate the CP-AMPAR by phosphorylating Ser831. Since CaMKII will have interrupted localized calcineurin activation, dephosphorylation of Ser845 required for LTD is less likely. Further research will be needed to explore CaMKII's regulation of the AMPAR described in this model. This potentiation is dependent on simultaneous norepinephrine and glutamate signaling, but leaves the synapse more sensitive to future stimulation[32-36] (Model for these interactions can be seen in Figure 3). Previous studies have shown that S845 is phosphorylated when beta-1-adrenergic receptor is in complex CP-AMPARs[37]. Both models of CP-AMPAR complexes in micro-domains with βAR are capable of stimulating the synapse in response to acute stress.

Summary

This review briefly describes how the balance of AMPAR expression may not only influence plasticity through membrane depolarization, but also as an important component of meta-plasticity. This model extends the importance of particular AMPARs past regulating the strength of synapses by shear numbers. AMPARs in these complexes could also function to prime the synapse for sensitivity key to initial learning and memory acquisition. Conceptually, these micro-domains collect the pro-

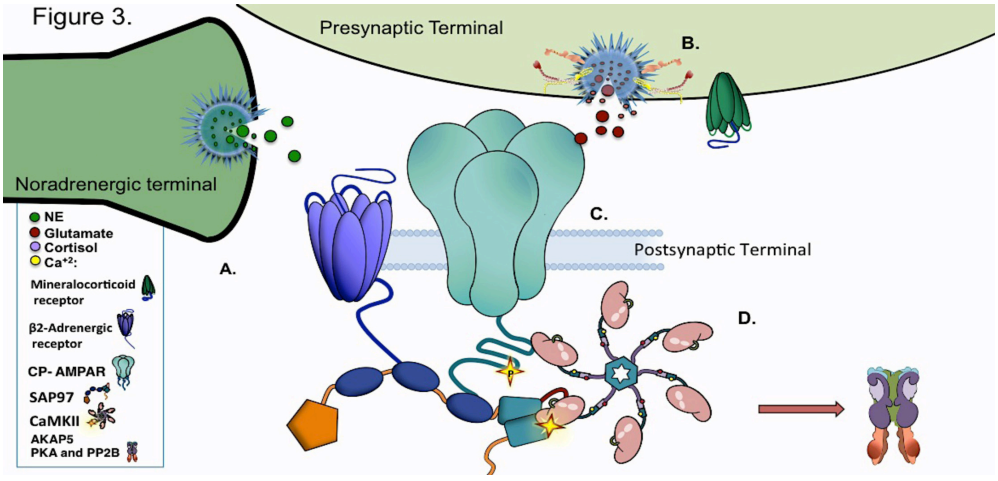

Figure 3. A) Noradrenergic signaling promoted by an acute stress response via the HPA axis, binds β_2AR and activates PKA through the cAMP pathway. PKA, localized to the CP-AMPAR by scaffolding proteins, AKAP150/79 and βSAP97-I3 phosphorylates S845 of the GluA1 subunit of the AMPAR to increase its open channel probability. B) The probability of increased calcium influx is also influenced by cortisol binding to the melanocorticoid receptors in the presynaptic membrane and up-regulating the release of glutamate into the synapse. C) Calcium influx through the CP-AMPAR then allows for calcium/calmodulin dependent activation of CaMKII, which can then phosphorylate SAP97-I3 to disrupt AKAP150/79 bound to SAP97-I3. D) This disruption removes PP2B, scaffolded by the AKAP, and prevents dephosphorylation of S845 and endocytosis of the CP-AMPAR. Finally CaMKII bound to SAP97-I3 is then in a perfect position to directly regulate the CP-AMPAR by phosphorylating GluA1 at S831 and increase the CP-AMPARs channel conductance. The net effect of this stress signal response is retention of a CP-AMPAR primed to have increased conductance and open channel probability. The CP-AMAPR then provides calcium signaling independent of membrane potential changes normally required for LTP, thus lowering the synaptic threshold for plasticity.

teins necessary for complex signaling to both be generated and retained. This model can explain how acute stress rapidly potentiates cell signaling and then retains molecular information to enhance memory. The receptors and proteins involved in this complex each show disrupted trafficking and expression when the organism endures prolonged stress. Future studies are needed to refine our understanding of how intricate protein-protein interactions in molecular complexes support synaptic sensitivity to modulate synaptic flexibility.

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Pay Attention: It's an ADHD Overview

Gwynne L. Davis

Attention deficit/hyperactivity disorder (ADHD) is the most commonly diagnosed childhood neuropsychiatric disorder. Currently, ADHD is diagnosed solely by behavioral observation, as there is no disease biomarker available. Disrupted dopamine (DA) signaling is often implicated in ADHD's underlying pathology. Furthermore, the most common pharmacotherapies for ADHD (Ritalin, or methylphenidate; Adderall, or amphetamine) exert their effects through the dopamine transporter, an important presynaptic regulator of DA homeostasis. Several mouse models exist for ADHD. These models provide the research field with valuable information, and each has its own strengths and weaknesses in helping to understand the underlying etiology of ADHD. A discussion of some the most popular ADHD models will be presented in this review, highlighting the documented biochemical and behavioral changes exhibited by each model and examining the relevance these changes may or may not hold for understanding ADHD. Importantly, all the models discussed and the models in the field lack something fundamentally important for understanding ADHD: construct validity. No current ADHD model is based on specific mutations found in ADHD patients, hindering progress in the understanding and treatment of ADHD.

Keywords *dopamine transporter, ADHD, SHR, DAT-KO, coding variant, construct validity*

Attention Deficit/Hyperactivity Disorder Introduction

Attention-Deficit/Hyperactivity Disorder (ADHD) is one of the most prevalent neurobehavioral disorders diagnosed in children. The disorder affects approximately 3-7% of school-aged children in the United States[1]. ADHD appears to be present in boys more frequently than girls with an average of a three-to-one bias[2]. This gender ratio changes depending on the subtype of ADHD and other factors such as setting. ADHD can be considered a spectrum disorder with a wide range of possible exophenotypes. The current diagnostic criteria for ADHD states that symptoms must be present before age 7 for at least 6 months in a manner that is "maladaptive and inconsistent with developmental level." The symptoms of ADHD are related to inattention, hyperactivity, and impulsivity, such as: difficulties in sustaining attention, organizational problems, excessive fidgeting or movement, impatience, risky behaviors, and many other symptoms. Based on the types of symptoms, ADHD diagnosis can be divided into three sub-categories: predominantly hyperactive/impulsive type, predominantly inattentive type, and combined type.

ADHD has a major effect fiscally on individuals and their families. Assuming that the rate of prevalence is 5%, in 2005 the economic cost of ADHD was between 36 and 52 billion dollars[3]. Those with ADHD, as adults, have a harder time maintaining jobs and decreased work performance[4-5]. Reportedly, those with ADHD are more likely to have a sick day within the past month and a 4-5% reduction in work productivity[5]. ADHD is also associated with an increased risk for other negative outcomes, such as an increased incidence in needing government support and higher instances of substance abuse[6-9]. Specifically, those who are not on medication correlate with having worse outcomes in terms of illicit polysubstance abuse[9]. As of 2007, 66.3% of those diagnosed with ADHD between ages 4-17 were reported to be on some sort of medication[10].

Diagnosis of ADHD is on the rise, with a 22% increase of diagnoses between 2003 and 2007[10]. There is no cure for ADHD, though several medications are available. Concerns about these medications have been raised because oftentimes they are psychostimulants, such as amphetamine and methylphenidate, with potential addictive properties and long-term negative effects. Because the cause of ADHD is unknown it is unclear how these psychostimulants are having their paradoxical calming effects on those with ADHD. The predominant theory is that ADHD thought caused by a disruption in the homeostasis of dopamine signaling. Thus, studying the dopamine system could provide an explanation for the behaviors seen in ADHD.

Brief dopamine overview

Dopamine (DA) is a neurotransmitter that is typically associated with the reward pathway or locomotor circuit, however, it also contributes to memory, learning, and cognitive performance[11-12]. DA interacts with a variety of receptors that are coupled with both stimulatory and inhibitory G-proteins. This allows DA modulation to cause either an increase or decrease in the post-synaptic neuron's excitability, depending upon the type of DA post-synaptic receptor present[13]. An important and necessary component

of DA signaling and homeostasis is the dopamine transporter (DAT). DAT is a Na^+/Cl^--dependent 12-transmembrane domain transporter found on dopaminergic neurons near the synapse. This transporter regulates the amount of DA present in the extracellular synapse by transporting it out of the synaptic cleft and back into the cytosol of the pre-synaptic neuron for re-use[14].

Because the psychostimulant therapies that are effective in treating ADHD are known to directly target DAT, several imaging studies have focused on the potential differences of DAT in ADHD populations[15]. The majority of these studies show increased DAT binding in ADHD populations, even with drug-naïve patients[16]. Volkow and colleagues, however, demonstrated decreased DAT binding in the left caudate. In the putamen, there was no difference in DAT binding between controls and those with ADHD, but a strong positive correlation was demonstrated between DAT binding with increased scores of inattention for both groups(with the ADHD correlation at higher levels of inattention but parallel to the control correlation)[17]. Despite the mixed results, these imaging studies indicate DAT is altered in patients with ADHD.

DA-associated ADHD genetics

ADHD has been reported to be one of the most heritable psychiatric disorders with reports of heritability in twin studies ranging from 0.76 to 0.9[18]. Genetic perturbations in the DA system have long been associated with or linked to ADHD. One in particular consistent throughout the literature is the presence of a variable number tandem repeat (VNTR) in the non-coding region of the 3' end of the DAT-1 allele. First prompted to look at DAT because of the manner in which ADHD is treated, Cook et al. identified a significant association between ADHD and a 10-copy VNTR. They identified 3 allele types that included VNTRs of three, nine, and ten copies[19]. Since this initial identification of the 10-copy VNTR region of the DAT1 gene, other studies have also looked at its association with ADHD, and it is thought to convey risk for the disorder[19-21]. Other genes encoding the various DA receptors have also been implicated in risk for ADHD. One of the most commonly implicated is the gene for DRD4[22-25]. Mutations in the DRD4 gene have been cited as the most consistent and replicated genetic findings in linking ADHD with the DA system[23]. This risk has been associated mainly with a seven-repeat VNTR in the third exon of the gene, affecting the third cytoplasmic loop of the receptor[26]. Mixed accounts have been reported for those with this genotype in terms of poorer cognitive performance on tasks of executive function[27-30]. There do seem to be changes in neural networks and activation patterns, though, including decreased activation and less coupling of neural networks during cognitive tests associated with this genotype[31]. Mutations in the DRD1 and DRD2 genes have also been implicated[18,32]. Studies have also indicated that differences in DA-associated proteins can increase risk for ADHD, such that one's COMT[a] genotype can affect performance on working memory tasks[33]. The large association of DA and DA-related genes and ADHD has led to the use of several rodent models that have perturbations in their DA-systems as models for ADHD.

DAT knock-out mouse

One of the most widely used models of ADHD is the DAT knock-out (DAT-KO) mouse. First reported in the mid-1990s, this mouse model has been popular for studying ADHD because it demonstrates several phenotypes associated with ADHD[34]. The most overt of these is its extreme hyperactivity in a novel environment—the overall activity level of DAT-KO animals is 5-6 times higher than that of WT mice in both the light and dark phase of the behavioral cycle. Initially these mice were created to better understand the role DAT plays in DA homeostasis, but it was rapidly adopted as an ADHD model.

It is thought that the hyperactivity of DAT-KOs is supported by the abnormally high levels of extracellular DA found in the striatum of this animal, which is a 100-fold greater ratio of extracellular to total DA levels in the DAT-KO mouse compared to wildtype[35]. This high level is thought to be caused by the slow DA clearance that occurs at 100 seconds versus 1 second, allowing extended time in the synapse[34]. Interestingly, the overall levels of both DA and tyrosine hydroxylase are drastically reduced to less than 5% and 10%, respectively, compared to wildtype[35]. This decrease is not due to any structural anomalies, as the terminals are intact and the number of DA neurons between genotypes is equivalent[35-36]. In addition, there is a large reduction in both D1 and D2 receptors[34,37].

Profound behavioral effects accompany these and other biochemical changes in the DAT-KO. In addition to the hyperactivity, DAT-KOs show impulsivity issues in a variety of situations. In one measure of impulsivity, using the cliff avoidance test, adult DAT-KOs displayed an impaired cliff avoidance reaction[38]. They had an increased incidence of ap-

a. Catechol-O-methyltransferase: enzyme that breaks down catecholamines in the synaptic cleft

proaching the edge of an elevated platform and extending out far enough such that they fell off, demonstrating an increase in impulsivity and risk-taking behaviors. Interestingly, this was ameliorated upon an injection of methylphenidate. Pre-adolescent DAT-KOs also showed increased impulsivity and risky behavior demonstrated by the number of times they dip their heads over an unprotected edge in an elevated plus-maze compared to their wild type counterparts[39]. Additionally, they have cognitive impairment, specifically in spatial memory, though this cognitive deficit seems to appear later in life, not being present in pre-adolescent DAT-KOs[39-41]. They also seem to be impaired socially, spending less time engaging in social investigation[42]. When they are being social, it tends toward being aggressive. Additionally, these mice spend more time performing stereotyped and perseverative behaviors suggested to result in a restricted and inflexible behavioral repertoire. This is interesting because social problems have been reported in the ADHD community[43]. DAT-KOs also have deficits in sensorimotor gating using pre-pulse inhibition as a measure[38,44].

To an extent, aspects of the DAT-KO model seem more akin to a schizophrenic model than to an ADHD model[45-46].This model has also been cited as a depression model, showing impairment in the forced swim test[47]. Having such an extreme disruption in the DA system results in behavioral deficits that extend beyond the scope of what one could reasonably link or associate with ADHD. It does not allow for a fine-tuned in-depth dissection of the disorder. A major caveat with this mouse model is that when there is a homozygous loss of function of DAT in humans, it results in infantile parkinsonism-dystonia, a severe and early-onset neurological disorder[48]. That being said, the DAT-KO mouse is used as both a face- and predictively-valid model of ADHD in that it shows some ADHD-like behaviors, and these behaviors can be mediated by the therapeutics used to treat ADHD.

Spontaneously Hypertensive Rat

Another well-studied ADHD model is the spontaneously hypertensive rat (SHR). An advantage to this model is the expansion of the behavioral paradigms that may be performed with this model. Rats are able to undergo more complex behavioral tests in a shorter training time period that allow for the observation of more cognitive-related tasks. This model is one of the genetic rat lines derived from selective inbreeding of Wistar rats[49]. These animals are hyperactive[50-51] and demonstrate impulsivity, attention impairments, and cognitive deficits[52-55]. As a result the SHR has been lauded as the most validated model of ADHD[52]. There are some major caveats to this statement. The SHR does display a number of symptoms of ADHD, but these can be inconsistent, depending on the task and which strain of rat is being used to represent the control. In addition to this, the SHR has mixed results as a predictive model of ADHD.

In some studies, psychostimulants help the SHR in attention-related tasks, where in others it does not[52,54,57-58]. Additionally, psychostimulants do not have a calming effect on the hyperlocomotion aspect of this model but rather potentiate the behavior[59]. Similar to the DAT-KOs, this model also displays sensorimotor deficits as measured by pre-pulse inhibition (PPI) studies[56,60]. Though studies linking PPI measures with ADHD show mixed results, the majority demonstrate that the ADHD population does not suffer from PPI deficits[61-63]. This seems especially true when the paradigm does not require sustained attention of the test subjects[63]. So these PPI deficits seem more indicative of a translational measure for schizophrenia, not ADHD.

What is most disconcerting about using the SHR as a model for ADHD is the lack of a solid control comparison. Unlike mouse research, there is no designated wild type rat and an ADHD rat. That leaves comparing the SHR to other strains that are deemed as normal. The Wistar-Koyoto (WKY) rat oftentimes is used as the main comparison in behavioral studies of the SHR. Strain differences are seen in performance tasks involving attention and impulsivity, with deficits arising in the SHR[52-54]. In some instances, these deficits are ameliorated by methylphenidate or amphetamine[52,54]. When third strains are added, however, the apparent face validity drastically changes. A good example of this is when Sprague-Dawley rats were included with WKY and SHR in behavioral tasks to probe timing and motivation. The results indicate the WKY strain is behaviorally similar to the SHRs, but none of the strains showed differential sensitivities to methylphenidate or amphetamine[57]. In a different study comparing attention and impulsivity between SH, WKY, and Wistar rats in a differential reinforcement of low-rate responding paradigm[b], SHRs were out-performed by the WKY but not by the Wistars. Additionally, in a 5-choice serial reaction time test[c], the attentional performances of all three strains were equivalent, but as the task went on, it was demonstrated that the Wistars actually made more impulsive choices that were then attenuated by methylphenidate[58]. Certain studies have implicated that amphetamine potenti-

b. The differential reinforcement of low-rate responding paradigm requires suppression of a behavioral response for a specific latency to receive a reward.

c. The 5-choice serial reaction time test requires a response to a light flash from 1 of 5 options to receive a food reward; accuracy of response is considered a measure of attention.

ates some of the ADHD behaviors seen in SHRs, including hyperlocomotion. Additionally, SHRs were demonstrated to have social deficits that were exacerbated by amphetamine[59]. Additionally, atypical antipsychotics ameliorated these symptoms, which have also been shown in other studies with this rat strain and PPI deficits[60].

With this model, however, there is a lack of understanding as to what is causing these behavioral changes. Studies have shown that SHRs have a down-regulation of the D4 receptor in the prefrontal cortex[56]. Additionally, they have an increase in DA efflux in the striatum, with a decrease in basal norepinephrine efflux in the prefrontal cortex[64]. They also have increased levels of DAT. It has been proposed that this strain has a hypernoradrenergic and hypodopaminergic system in the prefrontal cortex, leading to some of the behavioral deficits. However, what is not known currently is where these deficits originate. Is the initial insult in the DA system, the norepinephrine system, or somewhere else entirely? At the least, there seems to be a slight problem in using this rat strain in understanding the underlying etiology of ADHD.

Rare DAT variants and a construct valid model

While these models provide valuable information for the effects that disrupted components of the DA system can have at the behavioral level, they lack an important factor, construct validity. As of yet, there is no construct valid model of ADHD, resulting in potentially serious gaps in our knowledge in the underlying mechanisms of ADHD. With all the genetic studies linking components of the DA system to ADHD, one is faced with the non-trivial challenge of deciding which component to focus on in the development of a construct valid model. One issue with this is that many of the common variants in ADHD are associated with non-coding regions of the genome. This leads to a variety of issues when considering a construct valid model of ADHD, including the uncertainty of how non-coding regions function and the lack of conservation in these regions between humans and mice. Where does this leave ADHD research then? This leaves us with mutations associated with ADHD found in the coding regions, which are highly conserved across species. Specifically of interest is DAT because of its implicated role in ADHD risk[65]. There are no common variants found in DAT associated with ADHD, but there are a wide variety of documented rare coding variants[66]. Studying rare coding variants of DAT would provide unique insights into functionally relevant perturbations in DA functioning couched in genetic disruptions associated with the population of interest. Making a construct valid model of ADHD with DAT mutations may also provide valuable insight into how the current pharmacotherapies of ADHD are having their paradoxical calming effect, a line of inquiry that has long been pursued in the ADHD field. Of specific interest, a rare coding variant was discovered in DAT in two brothers with ADHD during a screen for DAT coding variants in a cohort of ADHD patients. This mutation is in the juxtamembrane junction of the 12-transmembrane domain and is a single-nucleotide polymorphism (SNP) that converts an alanine at the 559th amino acid position to a valine. This mutation results in a DAT that supports anomalous DA efflux. Interestingly, this efflux is ameliorated by amphetamine, methylphenidate, and cocaine[67]. This rare coding variant is important to the ADHD field because it provides a mechanism with which to help understand a potential ADHD risk. Making a mouse with this rare variant has the potential to provide the field with some unique insight into the etiology of ADHD.

Conclusion

With the high prevalence and poor outcomes of ADHD, progress in understanding the etiology is critical. While there are good models to elucidate what occurs when DA signaling is disrupted, none of them are directly linked with ADHD. A construct valid mouse model is critical to increase the understanding of the molecular and cellular aspects of ADHD and how such aspects affect neural networks and behavior. A construct valid mouse model derived from a risk allele of ADHD could prove invaluable to this area of research. Specifically, through the utilization of rare DAT coding variants found in the ADHD population insight could be gained to the inner workings of ADHD.

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Interleukin-6 Signaling in the Central Nervous System: Pointing the Finger at Trans-signaling in Neuroinflammatory-mediated Neurodegeneration

Franklin D. Echevarria

Cytokines are a class of immune-system-related signaling proteins used by cells to communicate with the external milieu. One particular cytokine, interleukin-6 (IL-6), is released by a variety of cells in response to multiple stressors. First characterized as a factor in B-cell maturation, IL-6 was shown to promote cell viability and differentiation of neurons in vitro, suggesting a role in the central nervous systems (CNS). In this review, the ramifications of removing or overexpressing IL-6 in models of CNS injury and infection will be discussed. Based on current literature, evidence suggests that IL-6 is involved in both neuroprotection and neurodegeneration. Interestingly, neurodegeneration seems specific to IL-6 trans-signaling, a form of IL-6 signaling that is more robust and often associated with chronic neuroinflammation.

Keywords *cytokines, IL-6, neuroprotection, neuroinflammation, neurodegeneration*

IL-6: The Basics of a Complicated Cytokine

The term cytokine represents a diverse group of small proteins used in cell signaling and are typically associated with an immune response[1]. One particular group of cytokines, known as the interleukin-6 (IL-6) family of cytokines, includes IL-6, IL-11, IL-27, IL-31, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), cardiotrophin-1(CTF-1), cardiotrophin-2 (CTF-2), and cardiotrophin-like cytokine factor-1 (CLCF-1)[2]. Members of this family bind to class I cytokine receptors on the plasma membrane[3]. Interestingly, these receptors contain no inherent signaling activity. To signal, IL-6 family members recruit a signal transducer protein known as glycoprotein-130 (gp130) and form a ligand/class I receptor/gp130 complex. Following formation of the complex, multiple signaling cascades can follow. The most prominent cascade involves phosphorylation of the tyrosine kinase JAK2, followed by subsequent phosphorylation of the transcription factor STAT3, which influences the transcription of genes involved in cell viability[4, 5](figure 1).

Of the IL-6 cytokine family, IL-6 is the founding member[2]. Characterized in 1985, IL-6 was implicated as a secreted factor in an acute immune response[6]. IL-6 was shown to prompt B-cell maturation[7], induce fever[8], and trigger acute phase protein release in the liver, all to promote healing and return the body to homeostasis[6]. In 1988, *in vitro* studies using the rat PC12 line of neural cells demonstrated that recombinant IL-6 also serves as a neurotrophic and differentiation factor in neurons[9, 10], indicating a potential role for IL-6 activity in the central nervous system (CNS). Since these initial studies, IL-6 activity has been connected to both maintaining CNS health and exacerbating neurodegenerative disease[2], suggesting that a delicate balance of IL-6 signaling is required for optimal CNS health.

IL-6 activity is facilitated through two pathways: classical and trans-signaling[11]. Classical signaling involves the membrane bound IL-6 receptor (mIL-6R), while trans-signaling uses a soluble form of the IL-6 receptor (sIL-6R). Unlike some soluble receptors that serve as antagonists to their specific ligand, sIL-6R performs as an agonist, sensitizing cells to IL-6 that do not express mIL-6R, but still express gp130[11, 12]. Synthesis of sIL-6R occurs by either alternative splicing of IL-6R mRNA or cleavage of mIL-6R by metalloproteases (i.e ADAM10, ADAM17)[13]. Interestingly, a soluble form of gp130 (sgp130) has also been characterized[14]. However, unlike sIL-6R, sgp130 serves as an antagonist by selectively binding sIL-6R and blocking IL-6 trans-signaling[14] (figure 1).

IL-6: CNS Expression and Actions in CNS Development

Following the discovery that IL-6 is capable of acting as a neural growth factor, questions became geared towards where IL-6 and its receptors were expressed in the CNS. Various *in vitro* studies indicate that neurons and glial cells are able to express IL-6, IL-6R, and gp130 to some degree[15-17]. Endothelial cells[18] of the CNS vasculature are only able to express IL-6 and gp130[19] (figure 1). *In vivo*, IL-6, IL-6R, and gp130 are expressed in the retina, striatum, hippocampus, hypothalamus, cortex and cerebellum[20-28]. Interestingly, gp130 expression is higher compared to IL-6 and IL-6R[28]. This is most likely due to gp130 being activated by more than

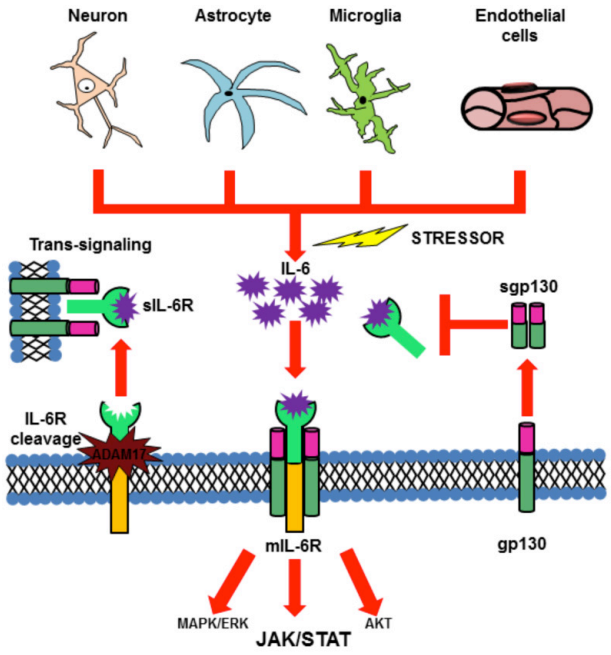

Figure 1. *How IL-6 signals in the CNS.* In response to stress, the cells in the CNS (neurons, astrocytes, microglia and endothelial cells) release IL-6. IL-6 binds to IL-6R followed by 2 copies of gp130. From there, multiple signaling pathways can commence. Also, there are two types of IL-6Rs: membrane bound (mIL-6R) and soluble form (sIL-6R). Signaling through sIL-6R is called trans-signaling and serves as an agonist to potentiate the IL-6 mediated response. A soluble form of gp130 also exists, which serves to inhibit trans-signaling.

one IL-6 family member.

The role of IL-6 in normal CNS processes is not well understood. Previously, it was mentioned that recombinant IL-6 promotes neuronal cell viability and differentiation *in vitro*[7,9]. *In vivo*, IL-6 knockout (IL-6 KO) mice show deficits in temperature sensitivity and a reduction in sensory compound action potential[29]. In the brain, IL-6 KO mice show a decrease in the neural progenitor cell population in the sub-ventricular zone and hippocampus[30], which is coupled with learning and memory deficits[31]. IL-6 KO mice also show behaviors of increased anxiety and aggression when exposed to new environments[32-34]. Overall, this suggests that IL-6 is involved in the development of certain neuron populations that regulate learning, memory, and responses to stressful stimuli.

Factors That Influence IL-6 Expression

Infection, injury and even basal activity are capable of influencing IL-6 expression in the CNS. *In vitro*, application of the bacterial endotoxin lipopolysaccharide (LPS) or the pro-inflammatory cytokines IL-1β and TNF-α induces IL-6 mRNA expression in glial cells[35-37] and neurons[37]. Up-regulated production of IL-6 in the CNS is also seen *in vivo* after peripheral injection of LPS in mice[38]. In animal models of CNS trauma, IL-6 elevation is seen after optic nerve crush[26, 39], brain ischemia[40-44], closed head injury[45] and peripheral nerve injury[46]. IL-6 elevation in both serum and CSF is also seen in people who have experienced traumatic brain injuries[47-50]. Interestingly, even acute physiological stressors are capable of influencing IL-6 expression. Depolarization of the plasma membrane induces IL-6 expression[51], suggesting that basic neuronal activity can induce IL-6 release. In addition, mechanical stressors such as elevations in atmospheric pressure can induce IL-6 expression in both glia and neurons[52, 53].

How glia and neurons induce IL-6 transcription in response to these stimuli is not fully understood. However, current evidence suggests that calcium (Ca^{2+}) influx plays a vital role. Removal of extracellular Ca^{2+} prevents pressure and LPS-dependent IL-6 secretion from microglia[52, 54]. In microglia exposed to elevated pressure, it also prevents NFκB, a transcription factor known to promote IL-6 expression, from translocating into the nucleus[52]. Removal of extracellular Ca^{2+} also prevented IL-6 expression in primary cortical neurons after depolarization[51]. However, IL-6 expression induced by membrane depolarization was not dependent on NFκB, indicating different stimuli are capable of inducing IL-6 expression in different manners[51]. Overall, these data indicate that multiple stressors are capable of inducing IL-6 expression. Interestingly, mechanisms involved in this process appear to be Ca^{2+}-dependent, an important concept since changes in intracellular Ca^{2+} are present in both neuroinflammation[55] and neurodegeneration[56].

IL-6 is a Potential Biomarker in Neurodegenerative Disease

Neurodegeneration is a broad term for the progressive loss of structure or function in neurons and is commonly associated with Alzheimer's disease, Huntington's disease, Parkinson's, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS) and glaucoma[57]. Recently, progression of neurodegenerative disease has been linked to chronic neuroinflammation, a state of inflammation in the CNS that involves constitutively active microglia, sustained cytokine production, and nervous tissue damage[57]. Due to its role in inflammation, it can be argued that IL-6 is a potential biomarker in this class of diseases. Indeed, elevation of IL-6 is seen in Huntington's[58, 59], MS[60-62], Parkinson's[63, 64], Alzheimer's[64], and glaucoma[65].

Alzheimer's is a form of dementia associated

with β-amyloid plaques in the brain[66]. It is suspected that β-amyloid plaques are involved in neurodegeneration[66]. IL-6 is elevated in serum and cerebrospinal spinal fluid (CSF) of Alzheimer's patients[64]. Interestingly, both *in vitro* and *in vivo* studies indicate that: 1) IL-6 elevation precedes plaque formation[67], 2) IL-6-positive glia are associated with β-amyloid plaques[68], and 3) IL-6 up-regulates the expression of the β-amyloid protein precursor[69]. Glaucoma is characterized by neurodegeneration of the optic nerve followed by progressive loss of retinal ganglion cells (RGCs)[27].The main modifiable risk factor is sensitivity to intraocular pressure (IOP)[27]. In patients with glaucoma, there is a significant increase in the levels of IL-6 in aqueous humor[65]. *In vitro* studies suggest that microglia are the main source of IL-6 in response to elevated pressure[70]. However, *in vivo*, 24 hours of IOP elevation induces IL-6 expression only in the RGC cell bodies and the optic nerve head of rats[39, 71]. Altogether, these data indicate that elevations in IL-6 could be an early indicator of CNS stress. Whether these elevations are significant enough to be used as reliable biomarkers remains to be seen.

IL-6: Neurotoxic or Neuroprotective in the CNS?

In basal conditions, exogenous IL-6 is capable of improving cell viability and promoting cell differentiation[9, 10]. When coupled with a specific stressor, pre-treatment with IL-6 prevents apoptosis in neural cells exposed to NMDA[72-74], elevated atmospheric pressure[53], increased intracellular calcium[75], and certain toxins[39, 76], further supporting the idea that IL-6 is neuroprotective. This hypothesis was further corroborated in models of CNS injury using IL-6 KO mice. In response to optic nerve crush, IL-6 KO mice show a decrease in the amount of axon regeneration from the injury site[39]. IL-6 KO mice also have a worsened CNS pathology after brain freeze lesion[77], brain ischemia[78], and dorsal column crush[79]. Conversely, IL-6 KO mice are resistant to sickness behavior[80], fever[81], and deficits in spatial memory[82] induced by LPS.

Interestingly, systemic or neuron-specific over-expression of IL-6 causes increased glial reactivity but no deficits in neuronal health[83]. On the other hand, mice with excessive production of IL-6 from astrocytes (GFAP-IL-6 mice) show not only increased glial cell reactivity[83], but reduced hippocampal neurogenesis[84], reduced long term potentiation in the dentate gyrus[85], an age-dependent increase in hippocampal cell death[83], and an age-related decrease in avoidance learning[86]. In response to focal brain injury however, GFAP-IL-6 mice show a more robust glial response, a decrease in apoptosis at the injury site and quicker healing time[87].

These studies suggest that IL-6 activity is both neuroprotective and neurotoxic. As a neuroprotective agent, IL-6 may function through the JAK/STAT pathway and promote cell viability[39]. As a neurotoxic agent, chronic exposure to IL-6 signaling, as seen in the GFAP-IL-6 mice, may lead to neuron dysfunction and subsequent neurodegeneration.

IL-6 Trans-signaling Facilitates Chronic Neuroinflammation

The ability for the CNS to initiate and terminate an acute neuroinflammatory response decreases in advanced age, resulting in a state of chronic inflammation[57]. Chronic inflammation is characterized by longstanding activation of microglia and continual release of inflammatory cytokines[57]. Unlike an acute neuroinflammatory response which promotes overall CNS health, chronic neuroinflammation re-

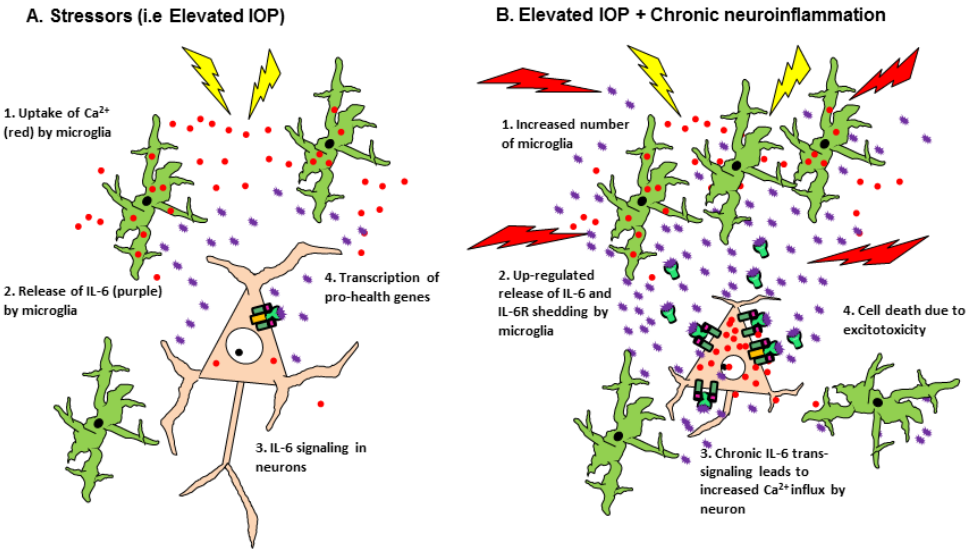

Figure 2. A) In response to stress, IL-6 activity is neuroprotective by promoting transcription of pro-health genes. B) In a chronic inflammatory microenvironment, increased IL-6 activity is present due to elevations in IL-6 trans-signaling, leading to a toxic buildup of intracellular [Ca^{2+}].

sults in gradual neuronal loss[57]. Interestingly, IL-6 may play a role in chronic neuroinflammation. Not only is there an age-related increase of IL-6 expression in both humans[88, 89] and mice[90], but microglia cultured from brains of aged mice show a more reactive phenotype and constitutively release more IL-6[90, 91].

Outside of the CNS, IL-6 trans-signaling is involved in the progression of diseases where chronic inflammation is thought to play a role including certain types of cancer, inflammatory bowel disease and arthritis[92]. Using a synthetic version of human soluble gp130 (sgp130) to specifically block IL-6 trans-signaling in mice, results from multiple studies suggest that blocking IL-6 trans-signaling improves the outcome of these diseases[93]. Whether the improvement is due to the sole presence of classical IL-6 signaling or due to decreased IL-6 signaling overall is not understood. Regardless, this discovery leads to one important question: Does blocking IL-6 trans-signaling attenuate chronic neuroinflammation and subsequent neurodegeneration?

As of now, only a few studies have shed light on this question. *In vitro*, sgp130 significantly reduces IL-6 release from both microglia and neurons exposed to LPS[94]. *In vivo*, intracranial injection of sgp130 reduces both LPS-induced sickness behavior and microglial IL-6 production in aged, but not young, mice[95]. In line with IL-6 trans-signaling, this study reports that: 1) microglia from aged mice express greater amounts of IL-6R than young mice[95], and 2) there is an age-related increase in hippocampal metalloprotease (i.e ADAM17) gene expression[95]. This suggests that IL-6R shedding from microglia is elevated in the aged brain, resulting in IL-6 trans-signaling-dependent chronic inflammation. Taken together, these data suggest that blocking IL-6 trans-signaling by sgp130 can mitigate chronic neuroinflammation and subsequent neuronal defects.

The Action of IL-6 is Dependent on the Microenvironment

Whether IL-6 functions in a neuroprotective or neurotoxic mechanism remains to be completely understood. Due to poor accessibility, studying the actions of IL-6 in the brain *in vivo* is difficult. Conversely, the retina is an excellent model due to its high accessibility. One particular neurodegenerative disease of the retina is glaucoma, an optic neuropathy that results in the progressive loss of retinal ganglion cells (RGCs) causing irreversible blindness. Like many other neurodegenerative diseases, age is a primary risk factor, while increased sensitivity to intraocular pressure is the only modifiable risk factor[96]. *In vitro* data from our lab show that recombinant IL-6 protects RGCs from pressure-induced death[53]. In addition, elevated pressure induces Ca^{2+}-dependent IL-6 production and release by microglia, suggesting that microglia release IL-6 to protect RGCs[70]. Interestingly, several studies have shown that glaucoma is associated with elevated levels of glutamate in the vitreous, suggesting a role of excitotoxicity in the disease[97-99].

In the CNS, one of the main responsibilities of microglia is to detect disturbances in the CNS microenvironment, and microglia are thus extremely sensitive to stressors such as elevated pressure and excess glutamate. It is postulated that in response to these stressors, microglia influx Ca^{2+} and subsequently release IL-6 to protect neurons (figure 2A). However, several reports show that chronic exposure to IL-6 increases Ca^{2+} influx through multiple glutamate receptors, resulting in toxic amounts of intracellular Ca^{2+} in neurons[100-102] (figure 2B). Therefore, it is our central hypothesis that in the CNS, the primary role of IL-6 is to protect neurons in response to noxious stimuli by initiating the transcription of genes that promote cell health. However, under periods of chronic neuroinflammation, extended IL-6 activity takes on a neurotoxic role. This may be due to increased IL-6 release from microglia and/or elevated cleavage of IL-6R, leading to an increase in IL-6 trans-signaling. This chronic IL-6 signaling subsequently causes prolonged accumulation of intracellular Ca^{2+} in neurons, resulting in apoptosis.

Conclusion

Communication between cell types is an important component to maintaining optimal health, both in the CNS and the entire body. IL-6, a cytokine released in response to neuronal stress, is capable of both protecting neurons and promoting their neurodegeneration. The manner in which it operates may be dependent on the state of the CNS microenvironment. As many neurodegenerative diseases are coupled with chronic neuroinflammation and IL-6 is a major player in inflammation, further understanding of the circumstances in which IL-6 signaling is either protective or harmful is crucial for its potential use as a therapeutic.

Further Information Rebecca Sappington's lab: https://my.vanderbilt.edu/sappingtonlab/

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An Integrated Neurocognitive Model of Strong Reciprocity

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The human species demonstrates a unique capacity for large-scale cooperation. This capacity has been linked to a theory of strong reciprocity, which posits that individuals, to varying degrees, show a willingness to engage in costly norm-enforcement in response to norm violations. Given the importance of cooperation to our modern society, the neurocognitive substrates of strong reciprocity have been subject to much investigation. However, these investigations have resulted in a division in the field. On one side is a body of research revealing the neural systems undergirding the phenomenon of the human proclivity towards norm enforcement. On the other is a body of research revealing the neural systems that support the evaluative and decision-making processes engaged by the actual decision to properly engage norm-enforcing behavior. Here, I review the contemporary knowledge provided by these two camps and present them as an integrated model.

Keywords *social decision-making, norm-enforcement, third-party punishment, altruism, fMRI, neuroscience and economics, neuroscience and law*

As the world becomes increasingly globalized, the fact that humans comfortably cooperate with genetically unrelated individuals becomes progressively more apparent. Our capacity as a species to engage in such cooperative behavior is one of the hallmarks that distinguish us in relation to other species, even those with high levels of social organization[1]. Anthropologists and biologists have long theorized that our cooperative nature depends on our ability to formulate social norms that establish boundaries of human interaction and exchange[2-3]. The extent to which these norms are culture specific is highly debated, though it is generally understood that some norms are highly dependent on culture and others seem to be much more ingrained in the human existence[4].

The legal maxim that "for every right, there is a remedy; where there is no remedy, there is no right" applies to social norms; where there is a norm, there is a punishment for violating that norm. And in the inverse, if there is no punishment, then there is no norm. Therefore, the phenomenon of social norms requires the existence of some form of intra-species punishing behavior. But who will be the punisher? There are two initially obvious answers to this question, though both are unsatisfactory. One is that the government must enforce punishment. However, inasmuch as we conceive of government as "by the people," this fails to account for the popular support of government-sanctioned punishment, thus the argument becomes circular. The second is that the individual who was harmed will enforce the norm. This second explanation fails to account for the fact that harmed individuals are frequently not in a position to enforce punishment and when they are, models have demonstrated that this kind of "direct reciprocity" does not sustain cooperation for large group sizes[5].

Herbert Gintis presented a solution for this problem in 2000 by identifying the phenomenon of "strong reciprocity"[a]; where disinterested parties (often referred to as third parties) would act as norm enforcers (i.e., strong reciprocators)[6]. Strong reciprocators are described as individuals "willing to sacrifice resources for rewarding fair and punishing unfair behavior even if this is costly and provides neither present nor future material rewards for the reciprocator"[7]. Data supported this theory as well; empirical models demonstrated that strong reciprocity could support maximum cooperation in environments where direct reciprocity models predicted no cooperation[8]. Behavioral studies have confirmed Gintis's theories, and now neurobiological models of this incredible phenomenon are being compiled. However, contemporary models have either focused on those systems that detect the norm-violation[9-12] or on those systems that evaluate and integrate the available information in order to formulate an appropriate response[13]. In this piece I review this literature and present an omnibus model of strong reciprocity behavior. Along the way I present new, testable, hypotheses that may explain lingering questions that existing models leave unanswered.

Behavioral studies support strong reciprocity

a. Strong Reciprocity: An observed phenomenon wherein individuals demonstrate a willingness to cooperate or punish that is not explained by a rational actor hypothesis.

Empirical models of strong reciprocity have been supported by laboratory observations of strong reciprocity. Economic games have been a commonly used, and widely accepted, method of revealing this behavior. Specifically, experimenters primarily employ the Ultimatum Game (UG)[b], though many other games are used as well.

The standard UG can best be described as a "take it or leave it" one-shot interaction between two individuals. One is given an endowment and is told to make a proposition to the other for splitting that endowment. The proposition can be whatever the proposer chooses. The offeree must choose to either take the offer or reject it. If they reject it, both parties get nothing. Even when the game is played in a one-shot manner—that is participants play, and know they play, a given opponent once—they will almost universally reject low offers. This behavior is characteristic of strong reciprocity due to the fact that the reciprocator is willing to forego an amount of money in order to punish another individual, even though there is no monetary or reputational benefit to doing so. Consistent with Gintis's theories, the availability of the punishment option drives up initial offers, maximizing cooperation and net outcomes for the group. The UG can be easily modified into a third-party variant by adding another individual (a "watcher") to the interaction. The watcher has the ability to punish either player, at a cost to the watcher's own endowment, following the two-party UG interaction. The presence of the watcher similarly improves cooperation[14]. Another game, called the Public Goods Game (PGG), is, arguably, a more ecologically valid economic game that is also used to model strong reciprocity. In brief, the PGG demonstrates that, when possible, some individuals will altruistically punish individuals who free-load off of public contributions. This also improves cooperation and net outcomes for the group[14].

Neurobiological Underpinnings – Detection of social norm violations

The act of strong reciprocity first necessitates that the individual become aware of a norm violation. Multiple studies have implicated the anterior insula (AI) as a key system involved in this alerting process[9,10,15]. Namely, in a UG paradigm, AI activity has been demonstrated to have a robust negative correlation with the size of the initial offer (stingy offers induce high AI activation) and AI activation will reliably predict whether the offeree engages in altruistic punishment[10].

These results are consistent with contemporary work on the AI, which has found the AI to be a critical area responsible for redirecting attention towards environmental stimuli that deviate from the expected course of events. In this sense it serves an alerting function for other brain regions[16]. This is consistent with multiple studies finding that AI is engaged by the presentation of an oddball visual or auditory stimulus, an oddball being perhaps analogous to a norm-violation. It must be noted, however, that detecting auditory and visual oddballs are distinct from the processes that must be engaged in the detection of social norm violations. The latter requiring a capacity to represent, to some form, the desires and intentions of others. That the insula, which has a relatively ancient phylogeny, is capable of detecting such violations is somewhat puzzling. One intriguing explanation as to how the human AI mediates this complex role is that it relies on a system mediated by Von Economo neurons (VENs). VENs have appeared in the AI in only the last 15 million years and are limited to humans and to the great apes, though they are present in far smaller numbers in the apes. VENs have been linked to fast human judgments related to evaluating social interactions, though the mechanism by which it mediates these interactions remains unknown[17]. Furthermore, immuno-cytochemical studies of VENs indicate a large presence of dopamine D3 receptors[17], which are linked to reward signaling[18]. This may, in part, explain the phenomenology of strong reciprocity in spite of the costs of action. Similar evidence that AI may be involved in the alerting and incentivizing process is that anterior insula projects to areas of the ventral striatum[19], which has been well-established as a critical player in reward-based decision-making[20].

Little is known about how the parameters of social norm compliance and violation are formed. For instance, in a UG with an initial $10 endowment, what neural system establishes and maintains that norm-complying behavior constitutes a contribution of $3-$4? The observation that, at least in the UG, norm estimations vary considerably across cultures[21] strongly indicates that the parameters are not established purely genetically but other evidence does indicate at least *some* genetic contribution[4]. Given the semantic nature of the norm information, it is likely that the norm representation is largely formed through social experience and maintains itself outside of the medial temporal lobe. However, no study has yet empirically tested this assertion. This hypothesis may be informed by studies that test strong reciprocity-like behavior in patients with medial temporal

b. Ultimatum Game: A common economic game designed to elicit strong reciprocity in subjects. Subjects are given the ability to accept or reject an offer made by another party. Rejection of the offer is always economically irrational but is commonly observed when the offer is below expected norms.

lobe damage.

Neurobiological Underpinnings – Acting on social norm violations

That humans can detect norm violations is a necessary element of strong reciprocity, but the defining element of the phenomenon is the willingness, if not the desire, to act upon the norm violation. Some have theorized that strong reciprocity, as it has been observed, co-opts domain general processes linked to emotional urges or impulses[22-24]. Others have hypothesized that norm enforcement is dependent on cognitive control of selfish desires, likely mediated by dorsolateral prefrontal cortex (DLPFC)[12]. However, recent research has cast substantial doubt on this theory. Particularly, Rand et al. presented convincing evidence that costly punishment, not selfishness, was the pre-potent, or impulsive, response[25]. This evidence supports the hypothesis that the decision to engage in altruistic punishment is an emotional response that overcomes the economically and evolutionarily (in the short-term, at least) rational act of foregoing punishment. If strong reciprocity is impulsive in nature, it is reasonable to hypothesize that such behavior derives from domain general systems that are responsible for trait impulsivity.

The systems responsible for emotion-driven impulsive behavior and its regulation are relatively well-characterized and center on corticolimbic structures. Corticolimbic–mediated impulses are signals that facilitate, oftentimes in an adaptive manner, attention and action to pertinent stimuli[26,27]. Meanwhile, the conscious perception of emotion is the phenomenological experience or awareness of these forces[28], with the strength of the emotion directly correlating with the physiological "need." It is well established that the amygdala plays a primary role in the development of emotional urges[26], and the amygdala has long been noted to be closely integrated with insular function[29-31]. Interestingly, insular connectivity with the amygdala is primarily associated with insula Von Economo neurons (VENs), which were discussed above for their possible role mediating the insula's involvement in alerting neural systems to deviations in social behavior from the norm[17]. Further, effective connectivity studies confirm what has long been noted: amygdala activity is strongly influenced by the insula[29]. Given this evidence, it's possible that the insula influences decision-making involving social norms based on reciprocal connections with amygdala, which translates norm deviation into an arousal response. The arousal response is likely mediated by neurons in the central nucleus, which augment cortical arousal via activation of cholinergic nuclei in the cholinergic basal forebrain[32]. Increased cortical arousal may be responsible for the impulsive response to engage in strong reciprocity via biasing of the response selection process in premotor and supplementary motor areas. This hypothesis is supported by a recent study that pharmacologically dampened amygdala activity and found a resulting substantial reduction in strong reciprocity, with no effect on the individual's perception of unfairness in relation to the stingy offers[33].

While unfair offers, or any perceived norm-violation for that matter, will likely trigger an insula and amygdala mediated augmentation of cortical arousal, it is clear that this response does not always result in action to correct the perception of unfairness. We can therefore infer what should be obvious, that emotional responses to unfair acts are modulated by other systems, systems that prevent maladaptive responses or emotional arousal to trivial violations. Data indicate that this occurs through a negative-feedback process involving the ventromedial prefrontal cortex (VMPFC). The VMPFC is believed to engage in this process by means of assigning action values to possible responses[34]. As part of this process, VMPFC is known to down-regulate amygdala-driven emotional arousal by exerting a top-down inhibitory influence in the amygdala in order to reign in emotionally driven, but maladaptive response options[35-37]. The hypothesis that VMPFC acts to down-regulate an initial bias towards punishment is strongly supported by two observations. First, VMPFC damage is linked to a significant increase in negative strong reciprocity in the UG. Second, as noted before, behavioral studies have observed that the strong reciprocity is more likely when a fast response is forced or when subjects are guided to place trust in their instincts. Inversely, when subjects are forced to delay their response or distrust their intuitions, altruistic punishment declines substantially[25].

Consistent with this mechanism, Cyders and Smith's model of a negative urgency disorder appears to be compatible with observations of strong negative reciprocity in only a segment of the population[38-40]. The authors define negative urgency as an emotion-based disposition to engage in rash action in response to negative affect, and they identify VMPFC (dys)function as the primary cause of the disorder. Given that strong reciprocity has been linked to rash action, negative affect, and is tempered by an intact VLPFC, the possibility that a behavioral predisposition to strong negative reciprocity is due to a so-called urgency disorder is worth consideration. Another interesting and compelling aspect to the possible explanation that urgency dis-

orders drive strong reciprocity is that Cyders and Smith also characterize a positive urgency disorder, which they define as a tendency to act rashly when experiencing positive emotion. A positive urgency trait may provide an explanation of positive strong reciprocity, which has been an understudied element to norm-enforcement research. Further evidence supporting the claim that urgency disorders may undergird strong reciprocity are empirical studies indicating that the urgency disorders, as with positive and negative strong reciprocity behavior, tend to not be expressed in the same segment of the population. Furthermore, the incidence of both occurs at similar rates[41]. Although classifying displays of strong reciprocity as indicative of a disorder seems to contradict claims that strong reciprocity is critical to our large scale cooperative society, this is not necessarily so. Several commentators have claimed that despite the possible contribution of strong reciprocity to large-scale cooperation, it is a maladaptive trait for the individual[41].

Neurobiological Underpinnings – Evaluating, integrating, and deciding on a response

If a norm violation is detected, and if that violation is sufficient to engage strong reciprocation on the part of the second or third party, the final network to be engaged is an evaluative and integrative decision-making network. This late-stage network is employed to weigh the particulars of the environmental stimuli and guide response selection. A proposal of the systems that mediate this evaluation, integration, and decision process has been put forth in a recent review by Buckholtz and Marois[13]. In their review, Buckholtz and Marois present a convincing hypothesis that this process is an evolutionary adaptation of other domain-general neural systems. They specifically focus on four regions: the DLPFC, medial prefrontal cortex (mPFC), Amygdala, and the temporoparietal junction (TPJ). Their model proposes that TPJ engages in mentalizing processes to encode information concerning the intent and blameworthiness of the act. The amygdala encodes affective arousal and functions as an affect-as-information system; a neural heuristic of the act's harm. They propose that mPFC integrates these two signals and coveys an integrated signal to DLPFC and this is used to bias punishment selection.

There is some data that support Buckholtz and Marois's intuitive hypothesis. Multiple studies have shown the TPJ to be an important component in assigning beliefs and intentions to others[42,43]. Further, Buckholtz et al. observed strong TPJ activation during a task where subjects were prompted to make punishment ratings after reading scenarios in which a particular actor was either responsible, or had diminished responsibility due to mitigating circumstances[44]. TPJ activity preceded activity in other regions and was most engaged in the diminished responsibility scenarios, which is consistent with the fact that the offender's mental state was most ambiguous in these scenarios. Studies have demonstrated that mPFC and amygdala demonstrate joint sensitivity to both harm severity and blameworthiness. The authors use this, and previous studies of amygdala and mPFC function, to support their hypothesis that the amygdala is acting to code harm through affect and that mPFC is formulating an integrated signal based on the actor's mental state and the resulting harm to arrive at an approximation of deserved punishment. Finally, the hypothesis that DLPFC is integrating and biasing signals from mPFC, is supported by findings that that DLPFC is engaged by the punishment response, and maximally engaged when participants choose to punish compared to no punishment selection[44].

Concluding Remarks

In this review I outlined an omnibus model of strong reciprocity that draws from multiple lines of research. This integrated model makes several propositions about the nature of strong reciprocity and claims to account for some previously unexplained observations in the literature. Altruistic behavior is incredibly complex, both from an evolutionary and neurobiological standpoint, and thus this model is hardly complete. Nevertheless, moving towards a more complete and harmonious understanding of this uniquely human behavior is important for forming a more comprehensive understanding of the foundations of human society.

Further Information

Lab website: http://www.psy.vanderbilt.edu/faculty/marois/LabHome.html

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This is the first paper to use neuroimaging to study third-party punishment. This article provides evidence that the TPJ is engaged in the evaluative processes associated with mental state evaluation, that activity in the amygdala may be correlated with the harm resulting from an individual's conduct, and that the DLPFC may be involved in the process of integrating these components in order to make a third-party punishment response.

Dopamine Neuron Physiology and Signaling – Why is DAT important?

Raajaram Gowrishankar

Dopamine (DA) signaling is dynamically regulated by multiple signals from the environment, thereby encoding information critical to the modulation of brain circuits subserving locomotion, reward and motivation. Perturbations in DA signaling have been attributed to multiple disorders such as Parkinson's disease, schizophrenia, bipolar disorder, addiction and ADHD. Furthermore, pharmacological manipulations in DA signaling have been used in the treatment of several of the aforementioned disorders. DA signaling is tightly controlled at the synapse by the presynaptic DA transporter (DAT), which transports DA into the neuron to maintain presynaptic DA stores. While current and past literature has focused extensively on the modulation of DA neuron activity and its relationship to behavior, the role of DAT in influencing DA neurotransmission and associated behaviors is not entirely understood. This review will summarize the intrinsic mechanisms involved in controlling DA neuron activity and how they impact behavior. It will underscore the importance of DAT in DA signaling highlighting key studies using the DAT knock-out mice and the multiple facets of DAT function in controlling DA signaling. Furthermore, it will also illustrate how aberrant DA signaling contributes to disease pathology highlighting studies on the characterization of a rare, missense mutation impacting the DAT coding sequence isolated from attention deficit hyperactivity disorder (ADHD) patients, the DAT Val559 variant.

Keywords *dopamine, dopamine signaling, tonic and phasic dopamine, dopamine transporter*

Since the discovery of DA as a functional neurotransmitter and not merely a precursor to norepinephrine biogenesis, the physiology of DA neurons and DA signaling have been the focus of much investigation[1]. Scientists have demonstrated that DA is at the crux of mediating behaviors key to locomotion, cognition, reward and motivation[2]. DA neurons undertake the important and rather complex task of integrating motor, sensory and cognitive information. DA is synthesized, packaged into vesicles by the vesicular monoamine transporter 2 (VMAT 2) and released predominantly by vesicular fusion mechanisms from DA neurons present in the ventral tegmental area (VTA), substantia nigra (SN) and the retrorubral field (RRF). DA release from the SN into the dorsal striatum (dStr), and VTA into the nucleus accumbens (NAcc) and prefrontal cortex (PFC)[3] respectively, initiates a cascade of downstream signaling events via activation of G protein-coupled receptors (D1 – D5 receptors)[4] ultimately resulting in the aforementioned behaviors. DA signaling is also controlled by signaling via somatodendritic and presynaptic D2 receptors that dampen DA neuron activity and inhibit DA release[5]. Reuptake of DA into the terminal by DAT serves as the primary mechanism for the termination of DA signaling at the synapse[6]. Thus, DA signaling is regulated at multiple steps ranging from presynaptic control of neuronal firing and DA release to the downstream signaling cascades following receptor activation, each equally important in contributing to the desired behavior.

It is therefore unsurprising that several studies point to a dysfunction in DA signaling as being causal to several neuropsychiatric disorders, ranging from the loss of nigrostriatal DA neurons in Parkinson's Disease to enhanced DAergic transmission in schizophrenia, bipolar disorder and ADHD. This review will focus on the presynaptic mechanisms involved in the initiation, sustenance and control of DA signaling – intrinsic mechanisms which influence DA neuron activity through the packaging and release of DA, the reuptake of DA by DAT to terminate DA signaling, and the modulation of DA neuron activity by DAT in both normal and diseased states.

Dopamine Neuron Physiology – From Activity to Behavior

The DA neurons of the SN and VTA are part of midbrain nuclei that encode important neural signals related to volition, reward and cognitive behavior. Evidence suggests that the SN DA neurons project to the dorsal striatum, forming the nigrostriatal DA pathway, while the VTA DA neurons project to the ventral striatum or NAcc, making up the mesolimbic pathway and to the medial prefrontal cortex (mPFC), forming the mesocortical pathway. This trichoto-

mous segregation has been suggested to be applicable to behavior as well, wherein the nigrostriatal pathway is involved in the control of movement, while the mesolimbic and mesocortical pathways are involved in motivation, reward and cognition. However, recent evidence points to an inter-mixing of SN and VTA DA neuron populations, wherein the more medial VTA DA neurons provide inputs to the ventral striatum, which establishes a feedback loop to more lateral DA neurons in the SN that project to the dorsal striatum[7].

Both SN and VTA DA neurons exhibit three distinct states of activity – 1) an inactive, hyperpolarized state, 2) a 'tonic', spontaneous, irregular, single spike form of activity and 3) a 'phasic', depolarization-dependent, burst-firing pattern of activity[8]. Approximately 50% of all DA neurons are not spontaneously active and are held at hyperpolarizing membrane potentials, likely due to GABAergic input from local GABAergic interneurons[9] or afferent input from the ventral pallidum, in the case of a subpopulation of VTA DA neurons[10]. The spontaneous firing of DA neurons is established via an intrinsic pacemaking mechanism, which is dependent on a hyperpolarization-activated cationic conductance (I_h)[11]. In contrast, burst-firing results in multiple spikes of activity and is depolarization-dependent[12], initiated via afferent control by cortical and brainstem nuclei[13-15]. Studies have demonstrated that phasic activity of DA neurons is dependent on NMDA receptors, as using an NMDAR antagonist inhibits depolarization and that this phasic activity is not sustained by glutamate alone[16]. The bursts of activity are often followed by a pause (after-hyperpolarization), followed by resumption of spontaneous activity[17].

The transition from tonic to phasic firing of VTA DA neurons is associated with reward-related cues, reward prediction errors and incentive salience[18-19]. Phasic firing is also depressed in response to aversive stimuli[20]. While lesion, pharmacological and transgenic studies have provided evidence for the role of the DA system in these behaviors, only recently have studies proven its causal role with spatiotemporal precision. To specifically interrogate the role of the DA neurons in reward-related behavior, Tsai and colleagues used a Cre-inducible adeno-associated viral vector (AAV) carrying a gene encoding the light activated cation channel channelrhodopsin-2 fused to an enhanced yellow fluorescent protein (ChR2-EYFP), in the antisense orientation. Stereotactic injection of this vector into the VTA of tyrosine hydroxylase(TH)::internal ribosomal entry site(IRES)::Cre transgenic mice results in ChR2-EYFP expression only in VTA DA neurons. Following this, the authors were able to reliably elicit tonic and phasic DA release with 1-5 Hz and 20 Hz or higher blue light pulses respectively. In order to test the role of selective activation of DA neurons in reward-related behaviors, the authors used conditioned place preference to behaviorally condition mice with either phasic or tonic optical stimulation. The authors found that phasic optical stimulation (50 Hz) sufficed to elicit behavioral conditioning and established place preference. The authors also found that tonic optical stimulation (1 Hz) elicited a trend towards place preference[21]. Since this pioneering study, several studies have interrogated the specific role of the DA system in the multiple phases of reward-seeking, the involvement of afferents in inhibiting this behavior and the contribution of its dysfunction to diseased states[22-26].

The role of tonic firing and tonic DA release in modulating behavior, however, has received little attention; although there have been a handful of studies dissecting the origin of tonic DA firing states[10] and the importance of tonic DA release[27]. An elegant model has also been proposed describing a putative role for tonic DA activity in the modulation of phasic firing via D2 autoreceptors, and its relevance to pathologies such as alcoholism, drug abuse and schizophrenia has also been proposed[28]. It is also unclear whether tonic DA release is vesicular or non-vesicular with studies proposing conflicting models. Studies on the manipulation of tonic DA to measure its effects on DA signaling and behavior have largely focused on genetic manipulations of DAT, as it can be argued that changes in DAT expression would result in alterations in tonic DA without the requirement of a change in phasic activity. Hence, for the purpose of this review, it is first necessary to gather a deeper understanding on DAT function before delving into how its alterations would relate to behavior.

Dopamine Transport and Dopamine Neurotransmission – Where does DAT fit in?

DA reuptake by DAT is the rate-limiting step in DA neurotransmission and provides a selective mechanism for the transport of DA into the presynaptic terminal. DAT is a member of the family of Na^+/Cl^--dependent transporters (solute carrier 6 or SLC6 family) and uses the transmembrane sodium (Na+) gradient (along with co-transport of chlorine (Cl-)) to transport DA across the membrane[29]. Following reuptake, DA is repackaged into vesicles by VMAT2. DAT thus plays an important role in regulating extracellular concentrations of DA and also in maintaining the readily-releasable pool of DA. In accordance with this, Giros and colleagues observed remarkable alterations in extracellular and intraneuronal DA levels in the DAT knockout (DAT KO) mice. The authors observed a fivefold increase in basal extracellular DA levels, and at the same time a marked reduction in intraneuronal DA stores that are dependent on reuptake through DAT. Furthermore, DAT KO mice also exhibited a fourfold decrease in the amplitude of evoked DA release[30].

Subsequent studies also showed that the lack of DAT-mediated DA recycling resulted in DA levels in the striatum of DAT KO mice to be dependent solely on the rate of ongoing DA synthesis[31]. Hence, DAT KO mice exhibit a state of elevated tonic DA while phasic DA is reduced due to depleted vesicular stores. Behaviorally, the elevation in tonic DA is manifested as spontaneous hyperactivity in both familiar and novel environments. Studies following this showed that DAT KO mice also exhibited deficits in the Morris Water Maze learning and memory test[32], impaired behavioral inhibition as shown using the 8-arm maze test[33], an elevation in reward-motivation as observed by a bias towards a positive tastant[34] and marked resistance to its extinction[35], thus highlighting the importance of DAT in DA signaling. DAT is also the primary target of widely abused psychostimulants such as cocaine and amphetamine (AMPH). Cocaine is a conventional DAT antagonist and blocks DA reuptake[36] whereas AMPH is a competitive DAT substrate, blocking DA uptake and is transported into the cell through DAT, thereby collapsing the vesicular pH gradient and depleting vesicular stores. This unpackaged cytoplasmic DA is expelled into the synapse via reverse transport through DAT and is referred to as DAT-mediated DA release[37-38]. Hence, based on this mechanism of action, it can be argued that while the elevation of tonic DA by cocaine is dependent on burst firing and phasic DA release, AMPH's actions are not coupled to phasic DA activity. The administration of either cocaine or AMPH in rodents has been shown to cause hyperlocomotion and stereotypy or repetitive behavior (at high doses), and increased motivation and reward-related behavior by an elevation in extracellular DA[39]. Giros and colleagues were instrumental in demonstrating that the hyperlocomotor responsivity to AMPH and cocaine is mediated through DAT, showing that this response was abolished in DAT KO mice.

Since the advent of the DAT KO mice, several strains of mice with varying degrees of DAT expression have been developed to further study the role of DAT in DA signaling. The DAT heterozygous mice (with 50% expression of DAT)[40], and to a certain extent the DAT siRNA mice (wherein DAT was knocked down to 60% expression via siRNA)[41], recapitulate many of the behavioral and neurochemical abnormalities of the DAT KO mice, albeit modestly. Of particular note are the DAT knockdown mice (DAT KD) where 90% of DAT expression was knocked down. The DAT KD mice express milder hyperactivity and impaired locomotion to AMPH[42], and in addition show enhanced motivation to reward stimuli[43]. Recent studies have used DAT KD mice as a model for elevated tonic DA and measured its effect on the learning and acquisition of reward-related behaviors showing that while DAT KD mice were not impaired in reward learning, they exhibited a lack of exploitation of this learning[44]. In addition to mice with decreased DAT expression, mice with enhanced expression of DAT have also been developed, which show hypoactivity in a novel environment, but no changes in response to DAT antagonists. Interestingly, a recent model of robust overexpression of DAT showed a threefold decrease in extracellular DA levels and a drastic increase in response to AMPH compared to wild type (WT) but not to cocaine, highlighting the importance of DAT in mediating AMPH-evoked DA release[45].

The action of psychostimulants on DAT and DA signaling has generated great interest in the field. While the action of cocaine has been fairly straightforward, recent studies have yielded rather complex and intriguing insights into the effects of AMPH on DAT and, as a result, on the DA system. The discovery that AMPH not only blocks DA reuptake but also promotes DAT-mediated DA efflux lead to the proposal that DAT exists in both inward and outward-facing states depending on the nature of DA transport[38]. Recent studies using transfected DAT in heterologous cell systems have also shown that the inward and outward states are independent of each other[46]. DAT-mediated DA release causes a gradual increase in tonic DA levels on a much larger timescale as compared to vesicular, phasic DA release and is dependent on intracellular calcium (Ca^{2+})[47] and an increase in intracellular $Na+$[48] (shown using transfected DAT in heterologous cell systems). However, Kahlig and colleagues demonstrated in HEK cells transfected with DAT that AMPH also mediates DA release in a complex and rare "channel-like" mode, releasing DA equivalent to that observed with phasic activity in the order of milliseconds. The authors suggest that this channel opening represents a unique conductance state open only under certain circumstances such as drug action in this case[49]. It has been proposed that such a conductance state could also arise owing to inward currents through DAT, coupled or uncoupled with the transport of substrate. In support of this theory, studies have shown the increase in excitability of cultured mesencephalic DA neurons in response to DA[50] and depolarization currents passing through DAT in cultured C. elegans DA neurons[51] that are blocked by DAT inhibitors. Hence, it can be argued that such a conductance state helps enhance the excitability of DA neurons resulting in DA release, but the physiological and functional relevance of DAT-mediated currents has been difficult to demonstrate *in vivo*.

The ability of DAT to efflux DA in response to AMPH has raised important questions. Does DAT release DA by itself under physiologically relevant conditions? And if so, what causes DAT-mediated DA efflux? Moreover, what does it contribute to? The existence of non-vesicular DA re-

lease was proposed over two decades ago, after the observation that a portion of DA released from SN DA neurons was non-vesicular in nature[52]. Anthony Grace proposed that a sub population of DA neurons exhibited burst firing-independent, tonic DA release under afferent control by glutamate and this established the tonic levels of extracellular DA[28], although evidence that this was mediated by DAT was scarce and not entirely convincing. Some years later however, Falkenburger and colleagues conducted an elegant study to specifically answer all the aforementioned questions. Their study sought to understand the role for dendritic DA, and for DAT expressed in the soma/cell bodies and dendrites of SN DA neurons. Using an *ex vivo* slice preparation, the authors observed that stimulation of the sub thalamic nucleus (STN), which affords afferent excitatory control of SN DA neurons resulted in the release of DA from the dendrites. They also observed that this DA release was DAT-dependent, as blockade of DAT abolished dendritic DA release, and non-vesicular, as extracellular Ca^{2+} depletion did not affect it. Furthermore, it was also shown that dendritic DA causes inhibition of DA neuron excitability via activation of somatodendritic D2 autoreceptors[53]. Following this study, Opazo and coleagues showed that DAT-mediated dendritic DA release is regulated by metabotropic glutamate receptors, coupled to PKC via the Gq-coupled signaling pathway[54]. Thus the authors have demonstrated a physiological role for DAT-mediated DA release in controlling DA neuron excitability via dendrodendritic inhibition. More recently, a voltammetric study measuring levels of endogenous DA *in vivo* in anesthetized rats has suggested the existence of two distinct states of DA neurotransmission in the dStr – 1) fast, arising from phasic DA release and 2) slow, arising from reverse transport of DA through DAT as this is sensitive to blockade by DAT antagonists. The authors also suggested that the DAT-mediated DA release results in tonic autoinhibition of DA neurons mediated by the D2 autoreceptors[55].

Hence, these studies establish a putative role for DAT in mediating an increase in tonic DA levels, and also in the maintenance of these levels, which could then possibly control phasic DA activity, both in the presynaptic terminals and the somatodendritic regions. Conversely, studies have also suggested that somatodendritic DA release is vesicular in nature mediating tonic autoinhibition of DA neuron activity via D2 autoreceptors using *ex vivo* slice preparations[56]. In order to reconcile the conflicting models, it is necessary that future studies focus on resolving the spatiotemporal properties of somatodendritic DA release and the properties they contribute to.

Dopamine Dysfunction and Disease – What's DAT all about?

Several studies have implicated aberrant DA signaling in the pathology of neuropsychiatric disorders. These include the loss of SN DAergic neurons resulting in the depletion of DA in Parkinson's Disease[57] and several genetic and imaging studies pointing to alterations in DA signaling in schizophrenia[58], bipolar disorder[59-60] and ADHD[61-62]. Furthermore, all drugs of abuse and alcohol directly or indirectly enhance DAergic neurotransmission; psychostimulants antagonize DAT, and alcohol enhances firing of DA neurons to increase the level of DA available both synaptically and extrasynaptically[39]. Control of DA signaling has also been harnessed in the treatment of these disorders – D2 receptor antagonists are used in the treatment of schizophrenia and psychosis[63] and DAT is the target of the most common pharmacological therapies used to treat ADHD[64], Adderall (amphetamines) and Ritalin (methylphenidate).

It is widely accepted that ADHD represents a case of functional hyperdopaminergia, whereby enhanced DA signaling contributes to the phenotypes such as hyperactivity, inattention and impulsivity observed in ADHD patients and ADHD-like rodent models[65]. Although the DAT KO mouse model has been the gold standard for understanding ADHD-like behavior, patients with a loss of function DAT allele exhibit Infantile Parkinsonian Dystonia, a syndrome that is in fact, a state of hypodopaminergia[66]. Hence, in an effort to clarify the DA hypothesis in ADHD, the Blakely lab identified several rare, heritable, functional, and highly penetrant DAT coding variants in many conserved sites in the DAT coding sequence from ADHD patients[67]. Mazei-Robison and colleagues undertook the *in vitro* characterization of one of these variants, the DAT Val559, and showed that when transfected into HEK 293 cells loaded with DA, the variant exhibited a DAT antagonist-sensitive, basal and voltage-dependent anomalous DA efflux (ADE). Furthermore, the authors observed that while AMPH caused DAT-mediated DA efflux in cells transfected with WT DAT, DA efflux was blocked by AMPH in cells transfected with DAT Val559[68]. A follow up study showed that DAT Val559-triggered ADE is sustained by the D2 receptor and mediated by a non-canonical signaling pathway involving Calcium Calmodulin Kinase II (CamKII)[69]. Previous work has shown that CamKII is essential for AMPH-evoked DAT-mediated DA release[70]. Hence, it is intriguing that a specific change in DAT function can lead to enhanced DA and possibly the phenotypes associated with ADHD. Current research in the Blakely lab has since focused on developing and characterizing a transgenic DAT Val559 knock-in mouse model, which would facilitate understanding of the profound changes in DA signaling related to ADHD.

Concluding Remarks

DA neurons play an essential role in relaying information necessary for locomotion, reward and cognition. While DAT plays an important role in controlling DA signaling via reuptake (the lack of which leads to profound changes in DA signaling), several studies have established a role for DAT in controlling the excitability of DA neurons by virtue of different conductance states of the transporter and its ability to release DA. With regards to its possible contribution to tonic DA levels, the DAT Val559 mouse model could afford us an exciting opportunity to dissect the role of tonic DA in the modulation of behaviors controlled by DA. Furthermore, it could also help in developing new hypotheses for the role of enhanced tonic DA in ADHD.

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Microglia Regulate Neurogenesis in the Developing, Adult, and Injured Brain

F. Edward Hickman

While the role of microglia as phagocytic nervous system immune cells has been well established, microglia have also been shown to play a unique role in regulating developmental apoptosis and neurogenesis. Developmental apoptosis causes about 50% of neurons to die before postnatal day 1 (P1), and these cell corpses must be eliminated to prevent a detrimental inflammatory response1-2. In addition, microglia have been shown to regulate neurogenesis in the adult subventricular zone (SVZ) and subgranular zone (SGZ). Only about half of these newborn neurons become integrated into the circuitry, and microglia are responsible for removing excess neurons as well as promoting the differentiation of new neurons3. Clearance of apoptotic cells, promotion of neurogenesis, and avoidance of inflammation are also crucial to maintain brain homeostasis following the cell death associated with a traumatic brain injury. This review will highlight how microglia regulate neurogenesis by directing apoptosis, clearing dead or dying cells and helping to maintain a precise balance of pro- and anti-inflammatory signals.

Keywords *microglia, development, neurogenesis, inflammation, phagocytosis, brain injury*

Origin of microglia

Microglia represent a large portion of the brain parenchymal area, between 6 and 12%[4-7] of all brain cells. While neurons, astrocytes, and oligodendrocytes all arise from the ectodermal layer during development, microglia arise from monocyte precursors originated from the mesodermal level. The prevailing hypothesis is that microglia come from early monocyte precursors residing in blood islands above the yolk sac[8], although there is some evidence for liver monocyte infiltration later[9] as well as bone marrow derived microglia that are able to colonize the brain after injury or compromise to the blood brain barrier[10]. Microglia from the mesodermal layer invade the developing nervous system around embryonic day 7.5(E7.5)[5-6,11] just before the onset of neurogenesis. Because they differentiate from a hematopoietic lineage like macrophages, microglia are professional phagocytes. In the resting, uninjured brain, microglia are considered to be "unactivated". However, this term is somewhat misleading because in this state they are actively surveying the CNS. In their unactivated state, they have a ramified morphology with extensive processes that are constantly probing for danger signals in the form of dead or dying cells, pathogens, axonal debris, or inflammatory cytokines. In contrast, activated microglia take on an amoeboid-like morphology, retracting their processes to facilitate phagocytosis[4]. Interestingly, this morphology is observed throughout CNS development as well, suggesting that microglia are primed to clear dead cells during times of significant apoptosis[12].

Like macrophages, microglia can be activated differentially, resulting in either pro or anti-inflammatory effects. M1-like activation is pro-inflammatory and detrimental to the brain. It leads to the production of other pro-inflammatory cytokines and is therefore cytotoxic. M2-like activation is neuroprotective and serves to promote cell growth by inhibiting inflammation with anti-inflammatory cytokines and growth factors. Among the receptors required to mediate M1-like microglial activation are TLR4, IL-4R, and INFγR [13-14]. This pro-inflammatory activation, which would be evident after traumatic brain injury (TBI) or cell necrosis, causes the microglia to express and secrete cytokines, such as IL-6, IL-1B, TNFα, and free radicals. In contrast, IL-4, IL-10, and IL-13 can activate microglia to be neuroprotective (M2-like). M2-activated microglia release trophic factors such as BDNF, NGF, and VEGF to promote neurogenesis[14-17]. Most anti-inflammatory cytokines also function to directly ameliorate any pro-inflammatory response by inhibiting their intracellular signaling cascades. There is some debate on the most consistent molecular markers of microglia in their active and inactive states, however Iba-1 seems to label all microglia while CD11b, CD68, and ED1 are used to distinguish M1-like activated microglia. In some forms of microglial activation, transcription of Mac1, F4/80, MCH, and FcR may also be increased[8]. Markers of M2-like neuroprotective activated microglia are arginase-1, and IL-1RA[4], however there are likely many more that have yet to be discovered.

Role of Microglia in Development

Microglia have multiple functions in the developing brain as varied as corpse clearance, promotion of cell death, and neuroprotection. During normal nervous system development, about half of all newborn neurons undergo programmed cell death rather than being incorporated into the circuitry. This massive cell death in the CNS occurs between E12 and E16 in mice, and by P1, very few dead cells are observed (by TUNEL and Annexin-V staining), suggesting that they are quickly and efficiently cleared by resident macrophages[18]. Apoptotic cells release "find-me" and "eat-me" signals to alert phagocytes, such as microglia, to clear them away; this aids in development and avoids inflammation[19]. The clearance of apoptotic cells during development relies on the presentation of phosphotidylserine (PtS), a primarily inner membrane leaf lipid, to the outside of the cell by scramblase enzymes. Microglia or other "professional" phagocytes express receptors to recognize PtS. While these receptors are largely unknown, the intracellular adaptor proteins involved, such as ELMO and Gulp, have been studied[20-21]. Binding of PtS to a receptor initiates a cascade of events that result in the actin cytoskeletal rearrangements within the microglia necessary for phagocytosis[20,22].

In addition to phagocytosis of apoptotic cells, pathogens and cellular debris, studies examining microglia localization and activity in various brain regions throughout development suggest that microglia instruct and assist in apoptosis. In the cerebellum, microglia clear granule cells to allow room for developing afferent fibers to synapse properly, in addition to aiding in the extensive cerebellar folding that must occur[23]. They have also been shown to instruct developmental apoptosis in the cerebellum by promoting apoptosis of Purkinje cells in organotypic slices through the release of superoxide, a reactive oxygen species[24]. In neonate hippocampal development, it has been demonstrated that microglia require the integrin CD11b (also a marker of classically activated microglia) acting on the immunoreceptor DAP12 to induce neuronal death[25]. The role of microglia has also been studied in retinal development. Microglial activation is shown to correspond with a release of NGF which ultimately results in a signaling cascade that triggers apoptosis of cholinergic retinal ganglion cells; this is possibly initiated through the interaction of NGF with $p75^{NTR}$ [20]. Microglia rapidly proliferate in the spinal cord around E12/13 in rats, a time that precedes the elimination of motor neurons, of which 90% will undergo apoptosis by E15. This delayed response to microglia and peripheral macrophages in the PNS represents a slightly different mechanism for regulation, in which the release of TNFα by early microglia primes the developing neurons for how they will respond to NGF later in development[26].

Recent studies on the developing primate cortex have also implicated microglia populations in clearing neural precursor cells[a] (NPCs). By genetically or pharmacologically blocking the activation of cortical microglia, an increase was observed in Pax6 and Trb2-positive NPCs. Conversely, *in utero* lipopolysaccharide (LPS) activation of microglia in the macaque monkey decreased the number of NPCs, suggesting that classically M1-like activated microglia are responsible for the decreasing pool of NPCs[4]. Microglia in the cortex can also provide neuroprotection. Developing cortical neurons, particularly in Layer V, require microglia-derived IGF1 as a trophic factor to maintain neuronal survival[27]. Microglia serve in an anti-inflammatory fashion by releasing regulatory factors such as NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), glial derived neurotrophic factor (GDNF), and neurotrophic cytokines. Additionally, release of macrophage colony stimulating factor by microglia supports neuron survival and neurite outgrowth in the developing cortex[28].

Microglia dynamics matches developmental time points and microenvironments at which they are most needed. Microglia are highly proliferative during development in order to clear the massive number of dying cells. The total number of PCNA+ (proliferating) microglia in rats has been shown to increase from 25% at E16 to almost 100% by P9 and then microglial cell numbers remain relatively constant, with only locally and minimally observed apoptosis throughout development[29]. The influence of microglia on oligodendrocyte development is crucial. Microglia have been observed in high concentration in developing axon tracts at a time when oligodendrocytes are differentiating and myelinating neurons, suggesting they are important for instructing this developmental process[30]. Microglia have also been shown to direct axonal migration within these tracts and clear debris from degenerated or unused axons during axon guidance and synaptic pruning[31].

Microglia in adult neurogenesis

Until seminal findings by Joseph Altman in the 1960s, neuroscientists thought the brain ceased creating new neurons by the time of birth. Altman, and many others since, have shown that there are areas of active neurogenesis in the adult mammalian brain, which contain neural stem cells able to differentiate into neurons, astrocytes, and oligodendrocytes[32]. Similar to development, in any region where neurogenesis is continuously occurring, there will be associated cell death. Thus these two processes are intimately linked. Microglia mediate this linkage by controlling neurogenesis and responding appropriately to a loss of neurons (i.e. apoptosis, TBI).

Neurogenesis in the adult subventricular zone (SVZ) along the lateral ventricles results in GABAergic interneu-

a. Neural precursor cell: partially differentiated, usually unipotent cell that has lost most or all of the stem cell multipotency and has committed to neuronal fate.

rons bound for the olfactory bulb. SVZ-born neuroblasts can either differentiate into olfactory bulb granule cells or periglomerular cells, depending on where they are born in the SVZ[3]. Doublecortin (DCX) positive neuroblasts migrate from the lateral ventricles to the olfactory bulb via the rostral migratory stream. Their continual birth and integration into the olfactory bulb circuitry and their survival are influenced by odor experiences, such as enriched odor exposure or odor discrimination learning[33]. Of the neuroblasts that migrate to the olfactory bulb, only 50% are integrated[3]. Microglia are responsible for clearing the neuroblasts that do not get integrated; this occurs before they arrive at the olfactory bulb[3].

The subgranular zone (SGZ) of the dentate gyrus of the hippocampus also creates new granule cells throughout life. Adult SGZ neurogenesis has been shown to be crucial for learning and memory tasks[33]. Recent evidence from Sierra, et al. has shown that excess newborn SGZ neurons in the adult hippocampus die within the first 4 days after birth and are cleared quickly and efficiently by resident microglia. Remarkably, these microglia appear to be "unactivated" which is evident by their morphology. These microglia are ramified, and phagocytosis occurs in pouches on terminal processes. This is in stark contrast to many studies that indicate that activation and the associated amoeboid morphology are required for phagocytosis. This phagocytic ability does not diminish in aged animals or animals that have experienced prior acute inflammation[34]. Microglia are able to influence apoptotic neurons because SGZ NPCs express Toll-like receptors, which respond to cytokines released by microglia in order to alter their proliferation capability[14].

The relationship between microglia and these neurogenic zones has been elucidated in several *in vitro* studies. Both SVZ and SGZ NPCs form non-adherent proliferating neurospheres[b] in culture. These neurospheres lose their ability to be multi-potent over time *in vitro* and are less likely to become neurons after continued passaging. Co-culturing primary microglia with these neurospheres rescues this attenuation in neural fate, suggesting that microglia residing in proliferative zones have a neuroprotective role keeping NPCs neurogenic, allowing for continued adult neurogenesis. In contrast, the normal loss of neurogenic activity matches the time scale of a decrease in microglia number caused by aging; the loss of microglia proliferation over time coincides with the observed decrease in neuroblasts born[35]. Several hypotheses have been formulated to explain this correlation between microglia and NPC differentiation during the late stages of neurogenesis. The loss could be a result of a terminal symmetric division of proliferating precursor cells that produces two neurons at the end of development and thus decreases the pool of NPCs[36]. Additionally, a surplus of stem cells can undergo apoptosis if they are not needed and culled by microglia at the end of development. It is also possible for these eliminated NPCs to have unknown defects that the microglia are able to recognize, leading to their specific clearance. A final hypothesis is that the microglia are releasing trophic factors to support NPC proliferation. A decrease in microglial number or a change in their activation state may cause a decrease in supporting factors. Cells may naturally undergo phagocytosis if they are not receiving proper trophic support. It has been shown that some pruning of NPCs by microglia does not require cell death to occur first but simply requires the expression of calreticulin (CRT) on viable or apoptotic neurons. CRT signals microglia through lipoprotein receptor-related protein (LRP), suggesting that these resident microglia can recognize and engulf healthy neurons before the intrinsic initiation of apoptosis and therefore have a much more complex role than simply clearing dead cells[37].

Similar to developmental neurogenesis and the above described *in vitro* neurosphere studies, there also appears to be a decrease in the number of committed neuroblasts as an animal ages. The decreased number of neuroblasts *in vivo* could be explained by the attenuation of neural differentiation or an increase in astrocyte promotion. IL-6 and LIF release by microglia has been shown to promote astrocyte differentiation via JAK/STAT pathway[38], similar to what is observed in development. Additionally, TGF-β is able to promote neurogenesis in NPC cultures suggesting that microglia are not simply pro- or anti-neurogenic, but regulate the careful balance of pro- and anti-inflammatory cytokines to control brain homeostasis[39].

Injury-induced inflammation and neurogenesis

Much of the research on the role of microglia in neurogenesis is conducted in models of brain injury or disease, which cause increases in pro-inflammatory cytokines or pathogens. In contrast to the developmental role of microglia, pro-inflammatory microglia are detrimental to neurogenesis. LPS activated microglia can instruct apoptosis by secreting TNFα, which promotes activation of BH-3 family member Puma via the NFκB pathway[40]. Blocking NFκB not only fails to activate Puma, but also causes an increase in survival of NPCs in an otherwise pro-inflammatory environment. Pro-inflammatory cytokines such as TNFα and IFNγ inhibit NPC proliferation in addition to NPC migration in models of brain inflammation[13]. The complete opposite effect observed between the response to IL-6 by NPCs in the SGZ and SVZ after hypoxia and ischemia injury exemplifies the complete dependence of microglia on type of activation and environmental specificity[41]. Additionally, IL-4-activated microglia showed a predisposition to cause oligodendrogenesis; in con-

b. Neurosphere: *ex vivo* primary cell preparation of non-adherent and proliferating progenitor cells

trast, the IFNγ activated microglia showed a bias towards directing neurogenesis[42] LPS- or IL1B-induced inflammation is detrimental to neurogenesis in the adult hippocampus; however activation of neuroprotective microglia and pharmacological anti-inflammatory treatment rescues this loss[43].

During times of brain inflammation, microglia are not just able to remove detrimental pathogens or dead cells and release pro- or anti-inflammatory cytokines. They also respond to attractive chemokines that guide them toward NPCs, aiding in the replenishment of neurons in an injured region[44]. The chemokine CXCL10 expressed in neurons in response to brain injury can activate microglia via CXCR. For example, the migration of microglia after entorhinal cortex lesion to damaged and inflamed brain regions is impaired in CXCR3 deficient mice[7,45]. A lack of migration would lead to a pro-inflammatory environment in the injured area, resulting in a reduction of new NPCs attracted to the injury site.

Alternative mechanism – are microglia the only cells at work?

This review focused on the plethora of data on the role of microglia in neurogenesis in both the developing and adult brain. Recent evidence has shown that macrophages and microglia are not the only cell type able to engulf dead cells. Satellite glial precursors in dorsal root ganglia can engulf apoptotic neurons during development to avoid detrimental necrosis and autoimmunity. A novel engulfment receptor, Jedi-1/PEAR1 is activated by eat-me signals to promote cytoskeletal rearrangement and clathrin-mediated endocytosis necessary for phagocytosis by satellite glial precursors[46-47]. Jedi-1, which was first discovered on macrophages, has been shown to possibly regulate macrophage/myeloid differentiation[48]. In the central nervous system, DCX+ NPCs have been shown to engulf apoptotic NPCs during both SVZ and SGZ adult neurogenesis. This engulfment is tightly linked to efficient neurogenesis. Blocking NPC engulfment by Annexin5 *in vivo* (binds to and blocks PtS) significantly decreases neurogenesis. These NPCs were shown to engulf dead NPCs that had been injected into the proliferative zones *in vivo*. This engulfment is mediated through unknown receptors acting through the adaptor ELMO; ELMO knockout mice show lower levels of neurogenesis[49]. Further elucidating the role of NPCs in neuroinflammation has potential therapeutic value. Several groups have shown the efficacy of transplanted NPCs in ameliorating T-cell activation and inflammatory signaling in immune cells and their protective effects from degeneration and promotion of regenerative immunological properties[50-52].

Clinical significance

The failure to clear cells that are undergoing apoptosis has pathological implications. Deficits in clearance lead to inflammation and a loss of neurogenesis, which has neuropathological implications, such as neurodegenerative diseases, depression, and anxiety[32]. Selective serotonin reuptake inhibitors (SSRIs) have been shown to ameliorate depression-like phenotypes by increasing adult neurogenesis. SSRIs, as

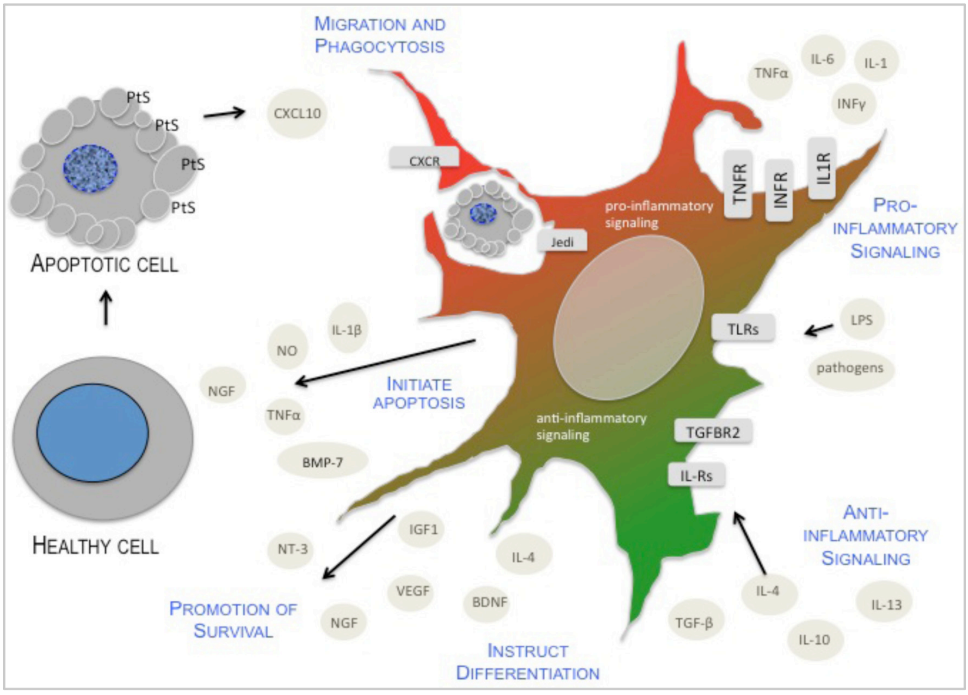

Figure 1. *Microglia have multiple functions in the brain to regulate neurogenesis.* Microglia can detect pro-inflammatory cytokines, such as TNFα, IL-6, IL-1, and INFγ. LPS or other pathogens can activate Toll-like receptors to mediate an inflammatory response. Often these cause the microglia to release factors such as IL1β, nitric oxide, NGF, BMPs to initiate apoptosis of neurons. Anti-inflammatory cytokines, such as TGFβ, IL-4, IL-10, and IL-13 cause microglia to instruct differentiation of precursors as well as promote the survival of these precursors. Chemokines released from dying cells can cause activated microglia to migrate to area of injury or cell death. Apoptotic cells express PtS, which may bind to receptors such as Jedi or MEGF10 to promote phagocytosis.

well as drugs that block inflammation, have proven to be promising drug candidates to counteract the microglial activation that often inhibits neurogenesis[16,53-54]. While the mechanisms by which SSRIs counteract depression and contribute to an increase in neurogenesis are largely unknown, one may hypothesize that SSRIs are able to act on microglia in a way that leads them to become less inflammatory and more pro-neurogenic. A more complete understanding of the mechanism by which microglia may instruct these pathologies will surely aid in our treatment options.

Whether cleared by microglia, NPCs, or traditional macrophages, the elimination of apoptotic or otherwise unneeded neurons is crucial to maintain brain homeostasis. Microglia are strategically positioned throughout the developing and adult brain to quickly and efficiently remove pathogens or dead cells, instruct developmental apoptosis, and to promote the survival of developing neurons that are needed for normal brain function.

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The Involvement of Superior Colliculus Neurons in Multisensory Processing and Orientation Behaviors

LeAnne R. Kurela

We are constantly bombarded with cues from our external environment in numerous sensory modalities. In order to properly perceive and interact with this environment, our brain must integrate this sensory information. While neurons located in various brain areas display this ability, the most-studied are located in the superior colliculus (SC), a highly conserved midbrain structure involved in both multisensory integration and orienting behaviors. Populations of neurons within the intermediate and deep layers of the SC have been separately studied for their roles in multisensory integration and saccade generation, however little research has explored the communication between the two systems. Previous studies have shown that multisensory stimuli increase accuracy and decrease reaction times of saccadic eye movements. However, evidence of a sensorimotor transform, an interaction between the sensory and motor systems to influence behaviors, remains controversial. Study of the neurophysiological underpinnings of multisensory integration and its role in saccade generation to multisensory targets is an important step in the determination of how sensory stimuli transform into the perception of, and interaction with, external events.

Keywords *multisensory, superior colliculus, electrophysiology, cat, primate, saccade*

The world is filled with sensory signals from multiple sensory modalities. In order to fully perceive this world, the brain must have the ability to process these sensory signals in conjunction with one another and integrate them to develop one single percept of the surrounding environment. Research into the phenomenon of multisensory integration[a] examines how these different sensory signals converge and are integrated by the nervous system, an imperative process for daily functioning. Multisensory processing is more efficient than individual sensory processing and is critical for everyday events. For example, the ability to understand speech in a noisy environment is aided by the visual information gained when concurrently viewing the face of the speaker[1]. Humans have greater accuracies and faster reaction times to multimodal stimuli compared to unimodal stimuli and the presence of a sound can change the perception and interpretation of ambiguous stimuli[2-5]. These behavioral benefits of multisensory integration are crucial for normal perception of and interaction with the external environment and dysfunction of such integration has been implicated in developmental disorders including those of the autism spectrum (ASD) and dyslexia[6-9].

It was historically accepted that input from each sensory modality is individually processed, converging only in higher-order cortical regions. It is now understood that this is not the case; convergence of sensory input occurs much earlier, at the single neuron level, in both early cortical and subcortical areas. These areas include primary sensory cortices[10-11], thalamic nuclei[12], the superior temporal sulcus (STS)[13] and ventrolateral prefrontal cortical (VLPFC) regions[14] in the primate as well as thalamic nuclei[15], anterior ectosylvian sulcus (AES) and rostral lateral suprasylvian cortex (rLS)[16-17] in the cat. One subcortical structure in which a multitude of multisensory research has been carried out is the SC, an area important in both sensory processing and orientation behaviors.

a. Multisensory integration: the ability to combine information from multiple sensory modalities in order to guide behavior

Multisensory integration in the SC

The study of multisensory integration has historically taken place within the SC of the cat. This is advantageous due to the amount of multisensory neurons within the structure; over 50% of the neurons found in the SC are responsive to multisensory stimuli[18-19]. However, much research is now conducted in non-human primate models, as the SC is conserved across these species and also contains multisensory neurons (Figure 1). A laminated structure, the SC is comprised of seven layers[20-21]. Based on morphology, connections and physiological properties, these can be divided into two functional zones: superficial and intermediate/deep. While the superficial layers are thought to be responsive to and re-

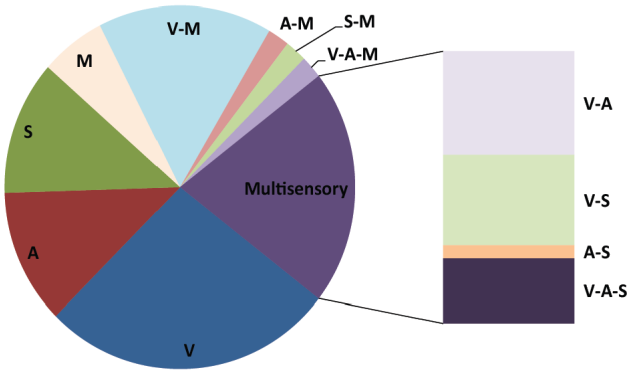

Figure 1. *Distribution of sensory and motor neurons in the intermediate/deep SC layers of the primate.* Motor, sensory and sensorimotor cells are all found in this location. V: visual-responsive; A: auditory-responsive; S: Somatosensory-responsive; M: motor-responsive (adapted from 64, 77, 82,83).

ceive inputs from only visual areas[22-23], the deep layers are the site of multisensory convergence, receiving inputs from visual, auditory, somatosensory and motor-related brain regions.

Neurons found within the deep SC layers are diverse both in size and inputs received[24]. Sensory inputs arise from visual[25-27], auditory[28-30], and somatosensory[31-32] brain areas while premotor neurons in the SC collect input from various motor-related structures[33-35]. Information received from these various regions form sensory topographies as determined by the receptive fields[b] (RFs) of the modality-responsive neurons, forming modality-specific maps that extend throughout the deep SC[36-38]. These topographic maps converge to form multisensory maps when cells respond to more than one sensory modality.

The overlap and interactions of these RFs contribute to the behavioral role of the SC in localization of sensory stimuli and orientation behaviors[39]. Previous studies report that SC-mediated eye movement orienting behaviors are facilitated under multisensory conditions, which is apparent via improvements in speed and accuracy of responses[40-41]. The loss of proper SC action results in deficits in normal responses and orientation behaviors[42-43]. The multisensory integration required for this improvement in behaviors occurs within individual neurons, following specific coding properties and principles by which information is integrated from various sensory modalities.

Particular combinations of stimuli are more or less salient than others, inducing neuronal responses that are increased or decreased relative to unisensory responses. Multisensory SC neurons exhibit these enhancements[c] and depressions[d] in response to combinations of sensory stimuli, and these response changes are central in guiding localization and orientation behaviors. Multisensory neurons in the SC can be divided into two classes: overt[e] and modulatory[f]. Both of these cell types engage in multisensory enhancement and depression in response to multimodal stimuli. This enhancement and depression of activity occurs based on the characteristics of the sensory stimuli presented. The characteristics and their subsequent influence on responses from multisensory SC neurons are captured in three principles of multisensory integration.

In order to properly perceive external events, the brain is required to determine which stimulus presentations are related to one another and which are not. Multisensory neurons use specific stimulus-related factors to make this determination, and these factors are explained in three principles of multisensory integration. The first is the spatial principle, in which a strong relationship is seen between the spatial proximity of presented stimuli to one another and the interactions that result in their combination; the closer two stimuli are in space, the more likely the multisensory stimulus results in neuronal response enhancement[44]. Conversely, spatially disparate stimulus presentations are more likely to result in response depressions. For example, when both a visual and an auditory stimulus are presented in spatial coincidence within a neuron's RF, the input is likely to produce a response enhancement. If, however, an auditory stimulus is presented within a neuron's RF and a visual stimulus is presented outside of the RF, a response depression is likely to occur. The temporal principle, similar to the spatial principle, explains that the largest gain from a multisensory stimulus presentation results when stimuli are in close temporal alignment[45]. A large temporal disparity has the ability to change a response enhancement into a response depression. The third principle, the principle of inverse effectiveness, states that the weaker the individual unisensory stimuli are in eliciting a neuronal response, the larger the gain in response magnitude from the combination of stimulus presentations[46]. As the ef-

b. Receptive field: a region in which the presentation of a stimulus alters neuronal firing

c. Response enhancement: response to a multisensory stimulus is statistically greater than the best unisensory response

d. Response depression: response to a multisensory stimulus is statistically lower than the best unisensory response

e. Overt neuron: neurons that show observable responses to more than one sensory modality

f. Modulatory neuron: neurons that show overt responses to one dominant sensory modality but have their responses modulated by the presentation of a sensory stimulus of a second modality

fectiveness of the individual unisensory stimuli increases, the gain in response from the multisensory stimulus decreases. These principles depict but a few of the factors that influence multisensory integration. Low neuronal spontaneous activity[47-48], proper sensory input from cortical regions[49-53] as well as sensory experience[54-56] are all critical for proper multisensory integration to occur in the SC. Multisensory integration is one of the key functional characteristics of neurons within the intermediate/deep SC; however it is not the only vital role. Some neurons within this structure are also critical for the generation of saccades[g].

SC involvement in saccadic eye movements

The generation of saccades requires the actions of many types of neurons, and some of these important neurons are located in the SC. Like multisensory neurons, saccade-related burst neurons and fixation neurons are found within the intermediate/deep layers of the SC, located more dorsally in this zone than the multisensory neurons[57]. Saccadic eye movements are represented in an orderly topographic map; fixation-related neurons are found at the most rostral pole[58-59]. Saccade-related burst and buildup premotor neurons encoding large movements are found in the caudal SC while those encoding small saccades are found more rostrally[60]. Fixation and saccade-related neurons are implicated in different steps important for saccade generation[61-63]. Fixation neurons are associated with the suppression of saccades via excitatory connections with neurons in the brainstem (Figure 2)[58,64-65]. Following the presentation of a signal to initiate a saccade, buildup premotor neurons in the SC exhibit response activity, increasing in their response as the generation of the saccade nears (Figure 2)[59,64]. These buildup neurons are involved in saccade preparation, exhibiting activity related to saccades of specific amplitude and direction[59,64]. At the time of buildup neuron activity increase, discharge rate of the fixation cells diminishes in anticipation of eye movement generation[58]. SC burst neurons begin to discharge closer to saccade initiation, producing a burst of activity approximately 25ms prior to the initiation of a saccade (Figure 2)[59,64]. The start of burst cell activity correlates with the termination of activity from fixation neurons, releasing inhibition and allowing for the movement of the eyes[58,64]. Proper activity of these cell types is imperative for normal saccadic eye movements, and this activity is not dependent upon intrinsic connections with neurons in the superficial layers of the SC[66].

Approximately 28% of neurons within the intermediate/deep SC discharge prior to a saccade (Figure 1)[64]. Neuronal populations discharge immediately before saccadic eye movements of specific distance and direction[67-69] and stimulation of these SC neurons evoke eye and head movements[70-73]. Neurons within the intermediate layers of the SC display activity necessary for regulating the production of saccadic eye movements, and the inactivation of these cells results in changes in speed, duration, frequency and trajectories of those movements[2,75]. Saccadic eye movements vary according to the sensory modality of the target; therefore, multisensory targets affect the dynamics of saccades[2-5,40-41].

Sensory-motor interactions within the SC

Saccades to multisensory targets are faster than those to unisensory targets[2]. It has been shown that saccades to audiovisual targets have the precision of visual saccades and the shorter latency of auditory saccades[4,41]. Additionally, spatially-aligned stimuli evoke the shortest reaction times in both non-

g. Saccade: an accurate, fast movement of the eyes in the same direction, used to precisely control gaze

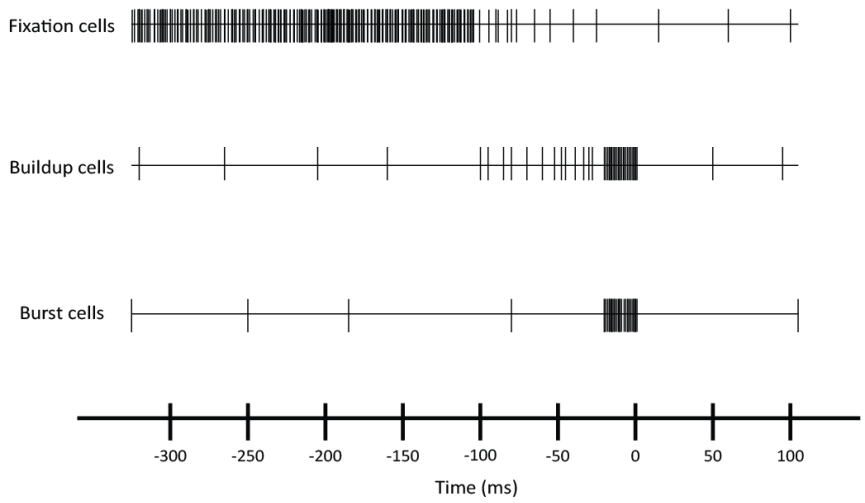

Figure 2. *Neuronal spiking patterns of SC motor-related cells prior to saccade initiation.* 0 ms: onset of saccadic eye movement (adapted from 58, 59, 64).

human primate models[40] and humans[76]. The presentation of an audiovisual target in temporal and spatial coincidence increases motor-related bursts in saccade-related burst neurons, supporting the idea that saccade-related neurons also have sensory processing abilities (Figure 1)[75,77-81]. While the same neuron can show both sensory and motor responses, the small amount of research focused on determining an interrelationship between the two response types has been conflicting.

Based on behavioral evidence, there may be an interaction, a sensorimotor transform, occurring between the saccade generation and sensory integration systems within the SC. However, little physiological research has been published to show this interaction. Intrinsic circuitry connecting motor neurons and multisensory neurons within the same layers has not been shown, and there is little research describing the interaction between sensory and motor responses within the same neurons. Future work in this field must involve examination of the physiological properties of individual sensorimotor cells within the SC to determine if there is an interaction between the sensory and motor activity that transforms the output of the cell and influences behavior. Likewise, much work is required to determine if the firing properties of multisensory neurons within the SC affect motor neurons in order to command gaze and saccadic movements to environmentally-relevant, multisensory targets.

Conclusion

Multisensory integration is an imperative process necessary for normal perception of external events. Integration of multimodal sensory inputs occurs at the individual neuron level and these neurons follow specific principles for integrating the input of multiple sensory modalities. Multisensory neurons are found in various brain areas and are highly prevalent within the SC. The SC is also involved in the generation of saccadic eye movements and it has been shown that these eye movements occur faster and more accurately with the presentation of multisensory compared to unisensory targets. This is ethologically relevant and advantageous; more salient events result in an orienting movement more often than less salient events. However behaviorally advantageous, little physiological evidence has been published to support the interaction of the saccadic and multisensory integration systems within the SC. Understanding the neurophysiological underpinnings of multisensory integration and its potential role in saccade generation to multisensory targets within the SC is crucial to determine if sensory stimuli transform into a perception of and interaction with external events. Comprehension of how this occurs normally is the first step to uncovering how these mechanisms are impaired in dysfunctional systems and finding methods or treatments to resolve the behavioral deficits caused by the inability to properly interact with the world due to deficits in multisensory integration.

Further Information: http://kc.vanderbilt.edu/multisensory/

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Redox Sensors as Regulators of Mitophagic Signaling: Novel Targets of Therapy for Ischemic Stroke

Britney N. Lizama-Manibusan

Depletion of oxygen and glucose during cerebral ischemia results in increased intracellular reactive oxygen species (ROS) formation and decreased levels of ATP. If these events proceed unchecked, neuronal death can occur within minutes and the inflammatory cascades surrounding the ischemic core can continue to promote death for days to weeks. Neurons have evolved strong adaptive features whereby mild ischemia promotes biochemical modifications such that cells are 'primed' to survive subsequent stresses. Surprisingly, activation of signaling pathways commonly associated with degeneration, including the production of ROS and activation of caspases1, 2 are required for the priming event to occur yet these stressful stimuli are held in check. Based on these preconditioning studies, as well as other research, we have increasingly come to understand that ROS do more than simply injure cells, but are also capable of activating proteins and transcription factors with essential roles in neuronal survival. Moreover, several redox-sensitive signaling pathways have been associated with the autophagic clearance of mitochondria, a process termed mitophagy, which can also lead to neuroadaptation by removing these injured organelles. This review will highlight the ways in which ROS act as discrete signaling molecules to activate pathways guiding mitochondrial dynamics and mitophagy that can promote cell survival following ischemic stress.

Keywords *antioxidants; HIF1α; mitochondria; mitophagy; p66shc; PINK1; reactive oxygen species*

Ischemic stroke encompasses 87% of all strokes in the United States and is the fourth most common cause of death in the country[3]. It is also the leading cause of long-term disability in adults[3]. During an ischemic stroke, a plaque or clot in a blood vessel results in loss of oxygen and glucose to the areas of the brain the vessel normally supplies causing neuronal excitotoxicity characterized by excessive glutamate release and hyperstimulation of NMDA receptors[4]. Once the plaque or clot has been removed or dissolved, re-introduction of oxygen promotes a second wave of ROS generation[5].

While prolonged ischemia is clearly damaging to neurons, strong evidence suggests that transient ischemic attack (TIA) prior to a more severe ischemic event can be neuroprotective[6]. This phenomenon, coined "ischemic preconditioning", can be elicited by a number of subtoxic stressors in addition to ischemia. Models of cerebral preconditioning share key features including: new protein synthesis, induction of heat shock proteins, activation of mitochondrial K_{ATP} channels and spatially and temporally limited activation of caspases[6]. We have increasingly come to appreciate that signaling pathways commonly associated with apoptosis and cell death can also be triggered during non-lethal, preconditioning events.

Our working model of preconditioning suggests that ROS function as spatially- and temporally-controlled signals, and while we have identified a variety of redox-sensitive molecules that contribute to protection, we still understand little regarding the role these molecules have on essential cellular processes including protein and organelle degradation. We hypothesize that the number and health of neuronal mitochondria plays an essential role in determining if neurons are preconditioned to withstand subsequent injury. This review focuses on the mechanisms by which neurons integrate energetic, and redox stress signaling to elicit engulfment of mitochondria in a process referred to as 'mitophagy' and how these events determine neural cell fate.

Reactive Oxygen Species in Neurons

The Double-Edged Sword of Aerobic Respiration

Eukaryotic cells rely on mitochondria for efficient generation of energy through the Krebs cycle and the electron transport chain (ETC). Indeed, eukaryotes contain an average of one billion ATP molecules, which turn over approximately three times per minute[7]. The central nervous system (CNS) relies heavily on aerobic respiration for ATP production as the brain utilizes over 20% of total oxygen respired, as well as 0.3-0.8 μmol of glucose per gram of weight per minute (μmol/g/min)[8], producing approximately 25-32 μmol/g/min of ATP. Notably, nearly 50% of this pool is required to main-

tain cellular ion homeostasis alone, thus underscoring the essential efficiency of ATP production by active mitochondria.

While production of energy-rich ATP by aerobic respiration is adaptive, it also produces free oxygen and nitrogen radicals. Not surprisingly, mitochondria produce the majority of ROS within neurons and approximately 3% of oxygen used for respiration captures electrons inefficiently resulting in the generation of superoxide anions[9]. Mitochondria are densely packed in dendrites and axon terminals and neurons have higher oxidative metabolic demands than glia and other cells within the CNS[10, 11]. Neurons continuously undergo biogenesis, fusion and fission of new mitochondria, and alterations in these pathways are associated with a host of neurological diseases[11-13].

Neurons Maintain a Highly Reducing Intracellular Environment to Combat ROS

One of the major ROS is superoxide anion (O_2^-), which is generated by electrons that escape the ETC through Complex I and Complex III and are transferred directly to oxygen[14-17]. Although O_2^- exhibits high reactivity; it is often short-lived and spontaneously dismutates to less reactive hydrogen peroxide (H_2O_2) with a rate constant of $8x10^4$ $M^{-1}sec^{-1}$. Alternatively O_2^- can be enzymatically neutralized by superoxide dismutases[18]. Hydroxyl radicals (OH), which are the most reactive oxygen radical known, are generated through reactions of metal ions with H_2O_2 or through fission of the oxygen bonds in H_2O_2. Hydroxyl radicals react almost immediately and indiscriminately with nearby molecules[9]. Indeed, these ROS are rabid electrophiles and remove electrons from proteins, DNA, and fatty acids[9]. Neurons have evolved a series of highly potent means to spatially and temporally regulate ROS. Glutathione (GSH) is the most abundant antioxidant, with concentrations reaching up to 18 nmol/mg of total protein in neurons[19], and directly reduces hydroxyl radicals and superoxides. During the process of reducing ROS, GSH is oxidized into glutathione disulfide (GSSG), and subsequently reduced back to GSH via glutathione reductase. The CNS sustains a higher GSH pool by increasing surface expression of the cysteine/glutamate exchanger (xCT), thereby increasing the availability of cysteine, which is the rate-limiting reagent in synthesizing GSH intracellularly[20].

Glutathione synthesis is energy dependent and involves two closely linked, enzymatically-controlled reactions. First, the amino acids glutamate and cysteine are combined by γ-glutamylcysteine synthetase. Next, GSH synthetase combines γ-glutamylcysteine with glycine to generate glutathione. Glutathione recycling is catalyzed by glutathione disulfide reductase, which uses reducing equivalents from NADPH to reconvert GSSG to 2GSH. As glutathione accumulates, it provides a negative feedback cue to limit further GSH synthesis[21]. All of these reactions require ATP either directly or indirectly[21].

The thioredoxin (Trx) proteins are present at intracellular concentrations of approximately 100 to 1000-fold less than GSH, but these proteins are also highly potent reducing agents[22]. Like GSH, Trx contains an oxidizable dithiol active site. Each member of the Trx family maintains specific intracellular localization to control local ROS flux.

Trx-1 is exclusively localized in the cytosol and nuclei, whereas Trx-2 is located solely within mitochondria. Trx proteins demonstrate rapid responses to specific stressors, such as H_2O_2, hypoxia and radiation[23-26]. Trx-1 translocates from the cytosol to the nucleus when its dithiol residues are oxidized where it can then regulate transcription factors such as hypoxia inducible factor 1 (HIF1), NF-κB, and p53[27, 28]. Trx-2, on the other hand, reduces H_2O_2 in mitochondria and is able to interact directly with members of the ETC, in addition to regulating the mitochondrial permeability transition pore (mPTP)[a]29 where strong evidence suggests that altering Trx levels promotes mPTP-dependent release of cytochrome c[30].

Other proteins, such as the superoxide dismutase (SOD) family of enzymes and catalases, act as neuronal antioxidants, but are less abundant. These enzymes utilize metal ion co-factors to reduce O_2^- into H_2O_2 and oxygen molecules and also demonstrate unique intracellular distribution and function. The copper/zinc SOD (CuZnSOD) is primarily cytoplasmic, while manganese SOD (MnSOD) is located within the mitochondria[31]. Overexpression of either of these antioxidant proteins decreases both infarct size and tissue damage in animal models of ischemia[32] and mutations in the SODs have been linked to familial forms of ALS[33, 34]. While the SOD enzymes utilize a metal ion co-factor, catalases utilize a heme group to reduce small molecules such as H_2O_2 and are particularly active within peroxisomes. In contrast to other antioxidant enzymes, very little catalases are active in mitochondria; thus H_2O_2 generated by mitochondria must diffuse to peroxisomes to be cleared in this manner[35]. In sum, by maintaining spatially localized antioxidant defenses, neurons can efficiently respond to increases in ROS.

ROS as Modifiers of Protein Structure and Function

Although ROS are often associated with DNA, protein, and lipid damage as well as cell death, it has increasingly become appreciated that they may also act as important mol-

a. The mPTP is a multi-protein complex that is gated by free nitrogen and oxygen radicals and controlled by pro- and anti-apoptotic proteins. Regulation of mPTP activity is poorly understood given that the components of neuronal mPTPs have not all been identified.

ecules for neuronal signaling[36-38]. ROS can reversibly modify specific amino acid residues to alter the structure and function of target proteins, which is akin to other post-translational modification mechanisms. For example, amino acid oxidation can influence the strength of protein-protein interaction and binding, alter the subcellular localization of a protein, or confer new properties and binding partners[39]. In addition, much like protein conformation determines the availability of a domain to be phosphorylated, amino acid structure and exposure determine the susceptibility of a protein to be modified by ROS. For example, proteins with exposed thiol groups, such as cysteine and methionine, are most easily oxidized by ROS due to the potent nucleophilic nature of thiols, and these oxidized thiol groups often proceed to form thiyl radicals or disulphides[9]. We speculate that localized ROS in neurons, specifically those produced by mitochondria, can modify redox-sensitive proteins to confer new functions relating to mitochondrial clearance and contributing to cell fate decisions during ischemic stress.

Controlled Clearance of Damaged Mitochondria through Mitophagy

Overview of Mitophagic Signaling

In the last five years, the role of autophagy – the controlled process of intracellular "self-eating" – has been show to play a critical and previously unappreciated role in neurodegeneration. During autophagy, signaling molecules called autophagy-related proteins (Atgs) initiate recruitment and formation of lipid bilayer vesicles (autophagosomes) that engulf targeted intracellular organelles and upon fusion with lysosomes, result in component degradation. Both neurons and glia undergo baseline autophagy essential for cell homeostasis[40, 41]. Therefore, perturbations in autophagic proteins can promote aberrant death that is most profound in CNS[42, 43]. Defects in mitophagy, in particular, have been linked to Parkinson's disease (PD) as animals with inherited mutations in PD genes have altered mitochondrial dynamics, cellular respiration, mitochondrial fission, fusion and mitophagy[44].

In spite of these compelling data, we have yet to develop a full understanding of the events which cause mitochondria to be engulfed and how endogenous signals like energetics and ROS combine with fusion and fission proteins to promote or halt mitophagy. We hypothesize that local, subcellular cues are essential to determining neuronal fate, and that under conditions of extreme ROS formation or energetic stress there are redundant opportunities for mitochondria to be recycled via mitophagy prior to neurons undergoing whole-scale autophagy. In support of this hypothesis, increasing GSH levels in yeast have been shown to inhibit mitophagy during glucose starvation, while not affecting overall rates of cellular autophagy. This suggests that mitophagy relies on redox-sensitive signaling elements[45].

Our understanding of the role of genetic mutations and cell-specific signaling including those related to mitochondrial function is guided by the PD literature. Mutations in two genes – PARK2 and PARK6 – are associated with early onset familial PD, and the protein products of these genes are now widely recognized regulators of mitophagy[46]. The product of the PARK6 gene is PTEN-inducible putative kinase-1 (PINK1), which is a serine-threonine kinase that is sequence-targeted to mitochondria. In healthy mitochondria, PINK1 is cleaved by proteases residing in the inter-membrane space and matrix and is targeted for degradation via the proteasome[47]. When mitochondria are stressed or damaged, mitochondrial membrane potential decreases – or depolarizes – thus reducing the activity of proteases such as PARL[48]. Depolarization thereby stabilizes PINK1 in the outer mitochondrial membrane[49], allowing PINK1 to phosphorylate target proteins via its kinase domain[50].

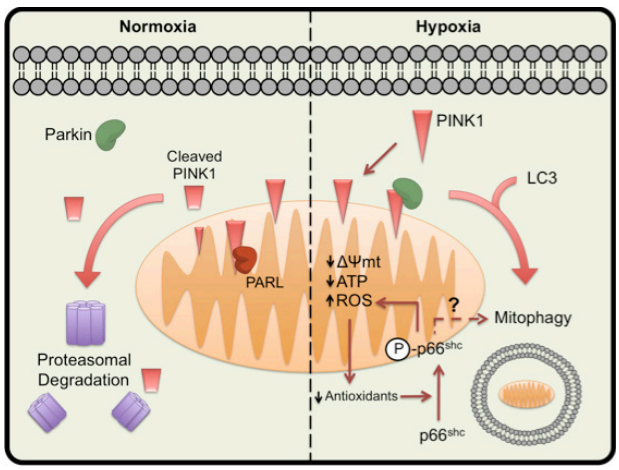

Figure 1. *PINK1 and p66^{shc} rapidly sense mitochondrial redox status and initiate mitophagy.* In healthy cells, PINK1 is cleaved and degraded via the proteasome. During hypoxia, mitochondria become depolarized, produce ROS, and decrease ATP output. PINK1 stabilizes on the outer membrane of damaged mitochondria and recruits Parkin, initiation LC3 recruitment and mitophagy. p66^{shc}, a redox-sensitive kinase, also rapidly localizes to stressed mitochondria, where it can regulate ROS levels and autophagy. Through these rapid sensors of ROS and mitochondrial health, mitophagy may be initiated duringischemic stress to enhance neuronal survival by removal of compromised mitochondria.

One of the known targets of PINK1 is the protein product of the PARK2 gene, Parkin, which functions as an E3-ubiquitin ligase. Recruitment of Parkin to mitochondria through PINK1 signaling is known to initiate mitophagy by activation of the mammalian homologue of Atg8, microtubule-associated protein 1A/1B-light chain 3 (LC3)[51] (summarized in Figure 1). Thus, the ability of PINK1 to recognize stressed mitochondria is crucial to the clearance of these damaged organelles by mitophagy.

Induction of Mitophagy During Ischemic Stress

Decreases in ATP and increases in ROS are mitochondrial events that are linked to neuronal mitophagy in both ischemia and PD based on genetic and biochemical data. In the context of preconditioning, mitophagy may be especially important, as clearance of compromised mitochondria following mild stress may promote a previously unappreciated form of neuroprotection, culling damaged mitochondria and leaving behind those most poised to survive subsequent stressors.

PINK1 & Parkin are Primary Initiators of Mitophagy

Given that PINK1 acts as a modulator of mitochondrial dynamics, and mutations in PINK1 are noted in PD, several *in vivo* and *in vitro* models of Parkinson's disease utilize mitochondrial uncouplers and ETC inhibitors to induce oxidative stress in dopaminergic neurons[52]. Functional PINK1 has been consistently shown to regulate mitochondrial dynamics in these models. For instance, neurons expressing a PINK1-kinase domain mutant exhibited similar mitochondrial morphology and function as wild-type (WT) PINK1 at baseline[52]. However, when stressed with the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP), neurons expressing mutant PINK1 exhibited greater oxidative stress and abnormal mitochondrial morphology indicative of damage and energetic dysfunction. Additionally, PINK1 depletion causes both inefficient calcium uptake and ATP production, which especially under high-energy demand, causes mitochondrial dysfunction which cannot be rescued by PINK1 mutants[b]53.

Ischemia in cardiomyocytes has been shown to be a potent means to induce PINK1-Parkin signaling and mitophagy and protection against subsequent stress[54]. In these studies, Parkin-KO mice were preconditioned with transient ischemia, followed by subsequent treatment with a more severe ischemic stress. Preconditioned WT mice exhibited reduced infarct sizes, yet preconditioned KO mice were found to have similar infarct sizes to that of naive mice[54]. This suggests that functional Parkin is necessary for cardiac ischemic preconditioning *in vivo* and that the mitophagic pathway may contribute to a neuroprotective phenotype. However, the investigators did not address PINK1 stabilization and upstream events at the mitochondria to allow Parkin recruitment.

While these studies are intriguing, they do not address the failure of Parkin KO animals to exhibit overt phenotypes related to neuronal and motor function loss, as seen in PINK1 KO animals. However, because key features of cardiac ischemic preconditioning are recapitulated in cerebral ischemic preconditioning, we hypothesize that PINK1/Parkin-mediated mitophagy is critical to determining neuronal fate in response to ischemia.

The Redox-sensor, p66^{shc}, is Essential for Neuroprotection and Mitochondrial Dynamics

While PINK1 localizes to mitochondria via its mitochondrial targeting sequence, one of the earlier redox-sensitive proteins discovered – p66^{shc} – becomes modified in response to oxidative stress and localizes to mitochondria upon activation. p66^{shc} is a serine kinase belonging to the ShcA family of proteins, which are adapter proteins studied extensively in cell signaling and exist in three isoforms – p46, p52, and p66[55]. p66^{shc} is unique in that it contains a glycine and proline-rich second collagen homology domain (CH2), allowing it to sense oxidative imbalance[56]. Phosphorylation within the CH2 domain, as well as reversible oxidation of cysteine 59 in the same domain, alters p66^{shc} conformation, promoting its redox activity. An additional modification that promotes p66^{shc} redox sensitivity is phosphorylation of serine 36 by protein kinase C-beta (PKC). This phosphorylation event allows rapid relocalization of p66^{shc} from the cytosol to mitochondria[57]. Indeed, PKC activation is a well-appreciated event in ischemic signaling and neuronal cell fate[58].

While the mechanisms that aid in interaction with the ETC remain elusive, once at the mitochondria, activated p66^{shc} oxidizes cytochrome c to promote H_2O_2 production and impair calcium buffering[59]. Our group has found that p66^{shc} is a critical mediator of energetic tone and autophagy in neuronal ischemic preconditioning. When p66^{shc} is inhibited, neurons contain higher concentrations of oxidized lipids and exhibit increased autophagosome formation[60]. Additionally, inhibition of p66^{shc} results in failure to upregulate the expression of chaperone proteins that can stabilize mitochondria and mitochondrial stress signaling[60]. Together,

b. Complete depletion of PINK1 in knockout (KO) animals, although not embryonic lethal, induces phenotypes consistent with PD pathophysiology, including progressive loss of dopaminergic neurons in the substantia nigra and motor function deficiency. In contrast, however, Parkin KO animals do not exhibit overt pathophysiological phenotypes, suggesting that compensatory mechanisms are involved to overcome loss of Parkin.

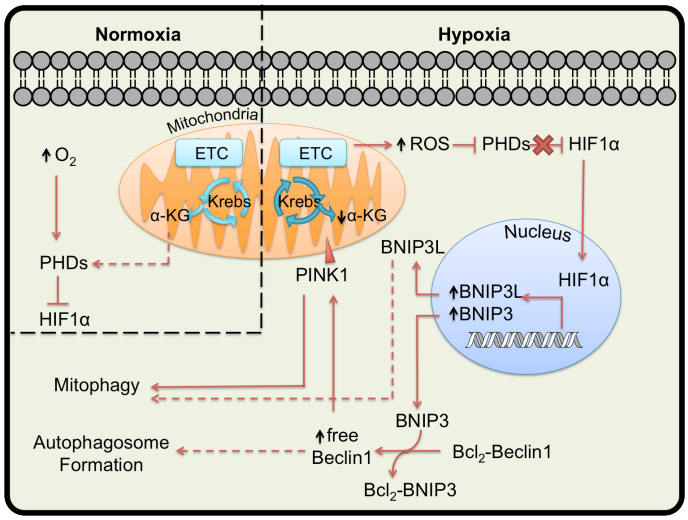

Figure 2. *HIF1α stabilizes during hypoxia and promotes pro-autophagic pathways.* Under ideal oxygen concentrations, HIF1α activity is inhibited through association with PHDs and subsequent degradation via the proteasome. Increases in ROS and decreases in Krebs cycle activity promote stabilization of HIF1α. This disassociation from PHDs allows HIF1α to translocate to the nucleus to upregulate the pro-survival genes BNIP3 and BNIP3L. Subsequent increases in these proteins have been associated with increased autophagosome formation and the induction of mitophagy. We hypothesize that these proteins are involved in the induction of mitophagy during ischemic preconditioning and adaptation to mild stressors.

these data reveal that, although activated p66^{shc} has been associated with cell death pathways, it is also a rapid responder to redox tone and a necessary component for protective pathways in response to mild ischemic stress. We suspect that because of its rapid localization to ROS-producing mitochondria and association with clearance of oxidized lipids, p66^{shc} may also be an important regulator of mitophagy.

HIF1α Promotes Autophagic Signaling During Hypoxia

Parallel literature from colleagues studying oxygen-sensing pathways suggest additional molecular sensors may play an important role in mitochondrial degradation in response to hypoxia and ischemia. In these studies, investigators found that prolyl hydroxylase domain-containing enzymes (PHDs) and hypoxia-inducible factors (HIFs)[61, 62] converge on molecular mediators of autophagy that have not yet been linked to p66^{shc}, PINK1, Parkin or the chaperone molecules and may, therefore, represent an independent sensor of stress and means to modify mitochondrial number and preconditioning.

Under normoxia, PHDs rapidly hydroxylate HIF1α subunits that then recruit an E3 ubiquitin ligase to ubiquitinate HIF1α, thereby forcing the proteasomal degradation of the protein[63]. Hypoxia inhibits PHD activity, promoting the stabilization of HIF1α, which can then promote the transcriptional upregulation of genes including vascular endothelial growth factor (VEGF), glucose transporters (GLUTs) and oxygen binding proteins among others[64].

In addition to its role as a transcription factor for genes encoding proteins involved in energy metabolism and vasculature remodeling, HIF1α exerts a role in autophagic and mitophagic signaling by activating BNIP3/BNIP3L, pro-survival members of the Bcl2 family. HIF1α stabilization promotes BNIP3 transcription, reducing the number of mitochondria via mitophagy[65, 66]. BNIP3 binding to Bcl2, releases Beclin1 from Bcl2 binding. Beclin1, the mammalian homologue of Atg6, has been studied as a pro-autophagic protein that stabilizes PINK1[67]. Taken together, these data suggest a model in which HIF1α increases expression of BNIP3, promoting free Beclin1, which would trigger mitophagy (Figure 2). Other research groups have provided additional evidence for BNIP3 and BNIP3L signaling as essential mediators of hypoxia-induced autophagy[68].

These roles of HIF1α are important considerations for our hypothesis that adaptive mitophagy occurs during ischemic stress to enhance neuronal survival even under conditions of high ROS. Yet, it is unclear whether HIF1α is able to promote mitophagy exclusively independent of autophagy, as this has yet to be investigated in mammalian models.

Clinical Implications for Targeting Regulators of Mitophagy

In the last two decades, several clinical trials have used antioxidants and free radical scavengers as a means to decrease the morbidity and mortality associated with ischemic stroke[69], with only modest improvement. One of the reasons for the limited success of these studies is that treatments in animal models are often administered at a predetermined time before or immediately after controlled ischemic onset. Stroke, however, is spontaneous and unforeseen, often meaning that patients do not reach a hospital quickly enough or within tested time windows for treatment. For example, less than 3% of patients qualify for receiving tissue plasminogen activator (tPA), a clot-buster and only FDA-approved drug for the treatment of ischemic stroke

(often given in conjunction with antioxidant treatment in animals), because it must be administered within 4.5 hours of stroke onset[c].

A second, larger concern with the use of antioxidants in the treatment of stroke is that preclinical data highlighted in this review strongly suggests that ROS are necessary for inducing protection, despite the historic association of ROS with toxicity and cell death, and thus overzealous use of ROS scavengers may hinder protective pathways under conditions of mild ischemia. Given that mitophagy is sensitive to oxygen and free radicals and downstream in cell stress signaling, targeting the molecular triggers of mitophagy directly may provide a more efficacious means to promote cell survival, as there are no FDA-approved pharmaceutical interventions specifically for preventing neurodegeneration post-stroke, and very limited therapies for further neuroprotection.

As genetic analysis is becoming more cost and time effective, researchers will be keenly interested in determining if polymorphisms or frank mutations in PINK1, Parkin, $p66^{shc}$, and HIF1α, may be present in individuals who are at-risk for poor outcomes following stroke which may account for the clinical heterogeneity in presentation.

Conclusion

Neurons are highly dependent on mitochondria for respiration and sufficient levels of ATP to maintain redox and energetic tone and synaptic transmission, and are particularly sensitive to damage from ischemia. This has led to an increasing desire to understand the cellular events that maintain redox and energetic homeostasis including high-efficiency antioxidants and redox and oxygen sensors. We hypothesize that controlled mitophagy to cull damaged mitochondria may be a previously unappreciated event that promotes cell survival and a target for therapeutic intervention given that several small molecule regulators of autophagy exist.

Further Information: McLaughlin Lab Website: http://www.mc.vanderbilt.edu/root/vumc.php?site=mclaughlinlab

c. Tissue Plasminogen Activator (tPA) is currently the only FDA-approved treatment for adult ischemic stroke patients. The approved window of treatment for intravenous tPA is 4.5 hours after symptom onset, while intra-arterial tPA may be given up to 6 hours after symptom onset directly to the site of the clot. Additional considerations for tPA administration include the fact that it is only effective for clearing blood clots and not other blood vessel obstructions, such as atherosclerotic plaques.

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The Impact of Cerebrovascular Function on Cognition in Normal Aging

Emily J. Mason

Aging negatively impacts both cerebrovascular function and cognition, even in the absence of underlying pathology. Although this is well established in the literature, the relationship between cerebral vasculature and cognitive ability has not yet been well characterized. Brain tissue is dependent upon proper perfusion for the delivery of oxygen and glucose, and it is, therefore, expected that even subtle impairments in brain vasculature will result in some loss of cognitive ability in healthy older adults. By understanding how cognition is impacted by perfusion in healthy aging, it will become much more straightforward to assess vascular components of pathological aging and dementia. Cerebrovascular function has been non-invasively studied through the assessment of global perfusion, region-specific perfusion, and vessel reactivity. In addition, neuropsychological and neurocognitive testing are able to investigate specific cognitive functions. In this review, the association between vascular factors and cognition will specifically be examined with regard to memory and executive function, as impairments in these domains are most commonly reported during healthy aging.

Keywords *Aging, Cognition, Cerebrovascular Function, Cerebral Blood Flow, Cerebrovascular Reactivity*

The world is aging at an unprecedented rate. In the next 50 years, the proportion of older adults in the population is expected to increase from 8 percent to 20 percent which will have a significant impact on economic growth, the labor market, and perhaps most crucially, the healthcare system[1]. Current models of social security and Medicare simply will not be able to cope with these changes, and unless work is done to improve the health and independence of older adults, the economic consequences will be disastrous. Intact cognitive ability is crucial to leading an independent life and remaining in the workforce. The decline in cognitive function during healthy aging has been well studied, and the specific cognitive domains that are typically affected include episodic memory[a] and higher-level executive function[2]. Episodic memory relies heavily on intact medial temporal lobe (MTL) function and is commonly assessed by examining a subject's ability to learn and retrieve material such as word lists or pictures. In contrast, executive function[bb] involves the prefrontal cortex (PFC) and is commonly assessed using tests of working memory, cognitive flexibility, verbal fluency, inhibition, and decision-making. Both episodic memory and executive function are critical to independent daily functioning.

Physiologically, healthy aging is often accompanied by subtle changes in blood vessels, such as hardening due to atherosclerosis, reduced or aberrant blood flow, and an impaired ability to respond to stimuli[3]. Eventually, these changes cause damage to the neurovascular unit[cc] leading to tissue ischemia, reactive oxygen species production, and cell death. Both the incidence of cerebrovascular disease and cognitive impairment increase with age, and it is important to understand how these factors interact. Reductions in cerebral perfusion[dd] likely contribute to cognitive impairment; however, this relationship has not yet been extensively examined. In support of this claim, cardiovascular disease risk factors such as hypertension, increased plasma homocysteine, obesity, poor diet, smoking, abnormal lipid profile, and high fasting glucose levels have all been associated with impairments in cognition[4-11]. Conversely, activities that improve cardiovascular health such as aerobic exercise are known to improve cognition[12-14]. Chronic hypoperfusion is known to cause atrophy of critical brain structures[15-18], and multiple theories implicate vascular dysfunction as a precursor to Alzheimer's disease[19,20]. Therefore, an in-depth exploration of the impact of vascular function on cognition in healthy aging may provide information that will help to develop mechanisms to slow or halt the progression of future neurodegenerative disease.

Understanding cerebral perfusion becomes par-

a. episodic memory: The conscious ability to recall an event.

b. executive function: An umbrella term used to describe a wide range of cognitive abilities. Although hotly debated, commonly listed executive functions are working memory, cognitive flexibility, verbal fluency, inhibition, and decision making.

c. neurovascular unit: The functional network of capillaries, neurons, and glia that control blood delivery to the brain.

d. perfusion: Rate of blood delivery to tissue, typically measured in mL blood/100 g tissue/minute.

ticularly important when considering the number of studies done using blood-oxygen level dependent functional magnetic resonance imaging (BOLD fMRI). Studies have shown both increased and decreased signal in older adults, and this result has been interpreted to be related to either impaired neuronal signaling or compensatory recruitment of additional brain regions, respectively[21]. However, because the BOLD signal is both qualitative and intrinsically blood-based, signal change could be due to a number of vascular parameters, independent of neuronal activity. Importantly, accurately characterizing the relationship between blood and cognition will significantly improve the interpretation of these studies. Historically, methods for understanding cerebral blood flow (CBF) such as transcranial Doppler imaging, positron emission tomography (PET), and single photon emission computed tomography have been limited by poor spatial resolution as well as the need for exogenous contrast. With improvements in MRI technology, it has become possible to assess vasculature non-invasively with resolutions of approximately 3-5 mm. Further, novel imaging such as arterial spin labeling (ASL) uses a radiofrequency pulse to "tag" inflowing blood water in a way that tissue perfusion can be quantified[22,23]. By manipulating labeling sequences, blood water can be separately tagged in the primary feeding cerebral arteries (carotid arteries, basilar artery) and can distinguish anterior and posterior perfusion territories (Figure 1). This technique, called vessel-selective or vessel-encoded ASL (VS-ASL, VE-ASL) is used to assess vessel collateralization and compensatory flow patterns and has been successfully applied in patients with cerebrovascular disease[23]. Unlike BOLD fMRI, these techniques provide insight into the role of vascular function quantitatively and not as the sum of several underlying factors.

It has been hypothesized that reductions in blood flow may be due to one of two causes: either brain tissue remains metabolically active and blood vessels are unable to react appropriately to stimuli, or brain tissue becomes hypometabolic resulting in a reduced perfusion requirement. To assess cerebrovascular reactivity (CVR), subjects are typically given vasodilators (CO_2, acetazolamide) and the increase in blood flow from baseline demonstrates cerebrovascular reserve. Deficiencies in CVR may be due to stiffening of the arterial wall and are thus independent of neuronal activity, although the contribution of neural factors can be inferred by examining the cerebral metabolic rate of oxygen and/or glucose ($CMRO_2$, CMRGlu respectively). This has typically been done *in vivo* using oxygen-15 (^{15}O) or fluorine-18 fluorodeoxyglucose (^{18}F-FDG) PET imaging[24]. Studies that have combined ASL and ^{18}F-FDG PET have shown that tissue metabolism is tightly correlated with CBF[25], although this finding does not clarify whether reduced metabolism leads to reduced CBF or vice versa.

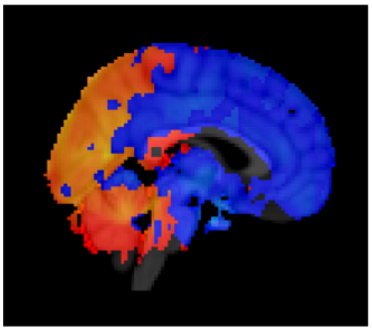

Figure 1. *Vessel-Encoded Arterial Spin Labeling (VE-ASL) can distinguish perfusion territories of the major feeding cerebral blood vessels.* Warm colors indicate the perfusion territory of the VBA; cool colors indicate the perfusion territory of the ICAs (courtesy of Manus Donahue).

Studies that have explored cognition in the context of vascular function are the subject of this review. Although there is still significant work to be done, it is clear that cerebrovascular reserve influences cognition. This may be especially true in memory and executive domains.

Memory and Vasculature

Episodic memory, which declines over the course of healthy aging, requires the complex interaction of synaptic integrity, glial support, and efficient energy metabolism in the hippocampus. Standard neuropsychological tests used to assess episodic memory require learning items and then recalling them after some delay. Examples include the Consortium to Establish a Registry for Alzheimer's disease (CERAD)[26], California Verbal Learning Test (CVLT)[27], and the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS)[28]. During memory encoding, information from the sensory and association cortices flows through the entorhinal cortex into the dentate gyrus via the perforant pathway (Figure 2). Mossy fibers from the dentate gyrus (DG) then synapse onto the CA3 region of the hippocampus. The CA3 region has an extensive auto-association network, which is believed to be responsible for the formation and temporary storage of episodic memories[29]. From there, the CA3 region projects to the CA1 region and the subiculum via the Schaffer collaterals, and the subiculum projects to the rest of the cortex. Any interruption in this flow of information may result in compromised memory. Normal aging results in hypometabolism in the DG at rest[30]. Interestingly, pattern separation, i.e. the ability to discriminate between two highly similar objects in memory, is dependent on the DG and is impaired during healthy aging[31]. Neither CBF nor CVR in the DG has been studied with regard to pattern separation ability, although BOLD

fMRI studies have indicated poor performance correlates with hyperactivation of the DG and CA3 during the task[32]. Knowledge of the individual roles of hippocampal subfields may provide an anatomically guided approach to pinpointing the influence of perfusion on specific hippocampal functions.

The hippocampus largely receives its blood supply through the posterior cerebral artery[39]. Under normal blood flow conditions, the posterior cerebral artery is supplied by the vertebrobasilar artery (VBA). Studies examining total cerebral blood flow have shown that global perfusion is associated with increased cognitive reserve[ee] [33,34], although few specific correlations between blood flow and memory have been reported. Global perfusion measurements are unlikely to reach significance due to their insensitivity to subtle changes; therefore regional alterations in blood flow may more accurately predict memory performance. As previously mentioned, VE-ASL is able to track the flow territory of the VBA. Flow territory asymmetry, i.e. vessels supplying tissues outside of their typical territory, is a surrogate measure of blood vessel collateralization. Healthy older adults that performed poorly on a memory task showed an increase in flow territory asymmetry[40]. Future work is encouraged to examine the relationship between memory performance and blood flow in the VBA, as well as vessel reactivity, using the VE-ASL technique.

Positive[35,36] and negative[37,38] correlations between MTL blood flow and performance on a memory task have been reported. Despite the seemingly contradictory nature of these results, both may be accurate. The relationship between blood flow and performance may depend on the stage of dysfunction. Early in the course of aging, there seems to be an imbalance in excitation and inhibition, which could cause hypermetabolism. Later, tissue damage due to excitotoxicity could result in a reduced requirement for oxygen and lead to hypoperfusion. Sensitive characterization of subjects both physically and cognitively would indicate the temporal pattern of hippocampal perfusion during cognitive decline.

Executive Function and Perfusion

Like episodic memory, executive function declines with age[2]. Executive function is an umbrella term referring to several higher-order cognitive processes. Neuropsychological tests of executive functioning typically assess working memory, cognitive flexibility, verbal fluency, inhibition, and decision-making. Because executive function heavily relies on intact brain networks rather than individual brain regions, it is difficult to determine the contributions of a particular anatomical area[42]. Case studies of individuals with frontal lobe lesions as well as PET and MRI studies have provided support for many aspects of executive function to be dependent on intact prefrontal lobe function[43,44]. While executive functions likely involve the interaction of multiple brain networks, some information on anatomical locations required for specific processes are beginning to emerge. The dorsolateral prefrontal cortex (DLPFC) is believed to be necessary for working memory, response selection, planning, and evaluating a choice[45]. The ventrolateral prefrontal cortex (VLPFC) is important for comparison of stimuli being held in memory, task switching, and reversal learning. Finally, the orbitofrontal cortex (OFC) is implicated in emotional processing of stimuli as well as distinguishing cue salience and reward. It should be noted that studies have reported lateralization of some executive functions in healthy young adults, although this lateralization disappears with age[46]. The Hemispheric Asymmetry Reduction in Older Adults model (HAROLD) predicts that older adults tend to activate more bilateral areas than young adults, potentially

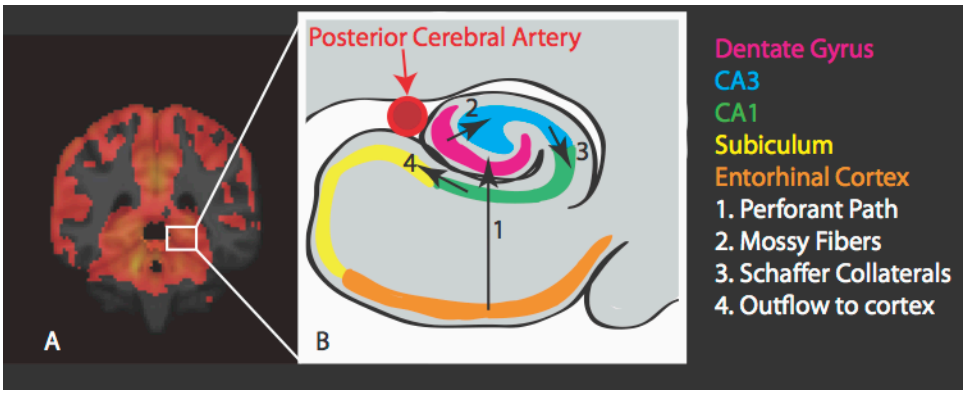

Figure 2. *Blood flow and hippocampal function.* A. Perfusion territory of the VBA, which includes the hippocampal formation. B. Schematic of signaling through the hippocampus.

e. cognitive reserve: The brain's ability to remain cognitively intact despite physiological damage, such as atrophy or plaque build-up.

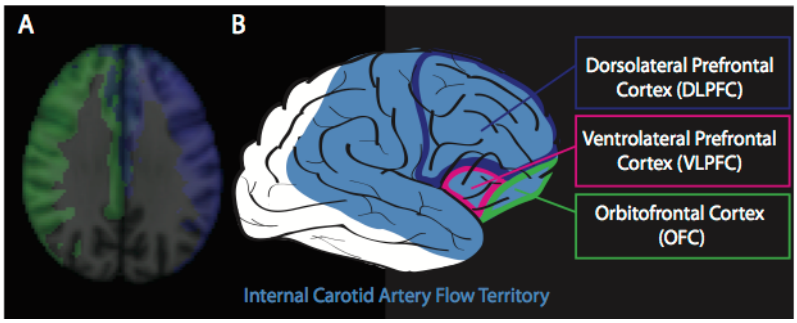

Figure 3. *Blood flow in the frontal cortexx.* A. VE-ASL image showing right (green) and left (blue) ICA perfusion territories (data courtesy of Manus Donahue, note this image is in conventional radiological orientation with the left side of the brain on the right side of the image and vice versa) B. Schematic of ICA perfusion territory and commonly reported anatomical sites of executive function.

to compensate for reduced signaling on one side[46]. Although the roles of specific anatomical regions in the frontal lobe are more difficult to identify than in the hippocampus, the impact of regional perfusion on specific functions may provide further information.

Recent work has reported that in healthy older adults, total CBF is positively correlated with information processing speed, attention, and other executive functions[17,18,47]. The frontal lobe is vascularized by the anterior and middle cerebral arteries, both of which receive their blood supply through the internal carotid arteries (ICA) (Figure 3). Currently, there are no studies examining executive function with vessel-specific perfusion, but ICA blood flow will likely be important. This becomes particularly true when considering potential lateralized functions in the frontal cortex.

Regional deficiencies in both CBF and CVR have been found throughout the brain, but particularly in the frontal cortex[48,49]. Although no study to date has examined the specific relationship between CVR and executive function, the spatial distribution of vessel reactivity deficits may be indicative of underlying pathology as Alzheimer's disease and vascular dementia show distinct patterns from normal aging[50-52]. Characterizing the impact of CVR and CBF on executive function may indicate a dissociation between healthy and pathologic aging.

Discussion

Several mechanisms for reduced cerebrovascular function have been proposed. Post-mortem studies reveal that the greatest detectable change with age in cerebral arteries and arterioles is thickening of the basement membrane as well as a loss of elasticity in the blood vessel due to increased collagen and decreased elastin[53,54]. This may be influenced by atherosclerosis or the beginning stages of amyloid angiopathy[51], both of which cause deposits to build up in the arterial wall. Some studies have indicated that blood vessels become more convoluted and tortuous with age, which might restrict blood flow[53]. Interestingly, Magnetic Resonance Angiography (MRA), studies have shown age-associated vessel tortuosity can be reduced by aerobic exercise[55,56]. Drugs are already on the market to improve vascular health, and using them to increase perfusion to key cerebral areas may one day allow for the slowing or prevention of significant cognitive decline with age.

There may also be impairments in signaling within the neurovascular unit. Vascular smooth muscle cells relax in response to nitric oxide (NO) and tighten in response to endothelin 1 (ET1), therefore the changes in vascular response may be caused by a reduction in NO and an increase in ET1 bioavailability[15,53,57]. These factors may induce basal hypoperfusion leading to degeneration of the neurovascular unit and a downward spiral of both vessel and neuronal function[50].

It is important to note that there are some limitations to the previously mentioned studies. For example, most studies did not take genetic status into account, and there are several common polymorphisms that may disproportionately affect cognition and/or vascular health. Caution must also be used in implying that impaired vascular health causes impaired cognition. It is possible that the relationships observed are due to subjects with higher cognitive abilities choosing to live more health consciously from a young age, and thus it is higher cognition that leads to better cardiovascular health and not vice versa[5]. Longitudinal studies across the lifespan would better establish this relationship.

Finally, the neuropsychological tests used in these studies are often designed to identify dementia and are not sensitive enough to find individual variation in the performance of healthy adults. The interaction between vascular factors and cognition should, therefore, be examined across subjects stratified by extremely sensitive neurocognitive tests designed specifically to understand aspects of healthy aging.

Conclusion

Impairments in vascular health seem to be mediating cognitive deficits, and there are a number of methods to determine how the two factors interact. By recognizing impairments in blood flow, evaluating vessel reactivity, and comparing these measurements to sensitive and accurate neurocognitive tests, the impact of cerebrovascular function on cognition in healthy aging may be understood. Novel MRI techniques will be able to explore these relationships not only in a regionally specific manner, but also in a vessel-specific manner, and these techniques will provide important insight into cerebrovascular function and cognition in healthy aging. Critically, these findings may indicate therapeutic targets that will slow or prevent cognitive dysfunction in aging and disease.

Further Information

For more information about the work done in the Ally Memory Lab, please consult our website: http://www.vanderbilt.edu/allylab/index2.html

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In Pursuit of Biomarkers of Alzheimer's Disease: Challenges and Opportunities in Tau and Amyloid Imaging

Richard McClure

Alzheimer's disease (AD) imaging research has contributed greatly to our understanding of the role Aβ plaques and tau-comprised neurofibrillary tangles (NFTs) play in AD pathology and has highlighted the potential utility of these hallmark lesions as biomarkers. As evidenced by the paucity of in vivo tau imaging studies to date, the majority of progress made in this field has been amyloid-centric. Although imaging of Aβ plaques has contributed significantly our understanding of AD pathogenesis, complimentary biomarkers such as tau must be validated and integrated into a more holistic diagnostic panel for the detection and study of AD. Here, we provide a review of the support for, and arguments against, employing PET, MR, and optical imaging technologies to track the deposition of Aβ plaques and NFTs in efforts to track AD progression.

Keywords *Alzheimer's Disease, Amyloid Imaging, Tau Imaging, Biomarkers*

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder recognized clinically as a progressive deterioration of short-term memory which expands to encompass increasingly global constituents of cognitive function[1]. Today, AD represents 50–70% of all cases of senile dementia and impacts nearly 35 million people worldwide. Notably, AD prevalence is higher in aging populations, with 1 in every 8 Americans over the age of 65 suffering from the disease. This statistic is most unsettling when coupled with the knowledge that as of 2011, the first members of the baby-boom generation celebrated 65 years of life. As a consequence of this demographic aging, based on current trends, 20% of the American population or 71 million individuals will reach the typical age of AD onset by 2030[2]. Notably, demographic aging is being mirrored in virtually all developing nations worldwide and thus threatens to compound the socioeconomic and public health calamity AD already represents to mankind.

As a result of these projections, the impetus to identify and characterize relevant biomarkers for AD has never been greater. Once identified, AD biomarkers may be utilized in three distinct applications: (i) as a diagnostic marker to aid in the early diagnosis of AD in clinically representative populations, (ii) as a classificatory marker capable of distinguishing AD from dementia subtypes that have similar clinical presentations, and (iii) as a prognostic marker capable of predicting disease progression with or without therapeutic intervention[3]. Here, we review the contributions of amyloid-β (Aβ) plaques and tau-comprised neurofibrillary tangles (NFTs) to the development of the cognitive deficits observed in AD and evaluate the potential of these two pathological hallmarks to serve as biomarkers. Current and future studies aimed at leveraging positron emission tomography (PET), magnetic resonance (MR) and optical-based imaging methods to validate Aβ plaques and NFTs as biomarkers of AD are discussed within the context of an increasingly refined model of AD pathogenesis.

The Role of Aβ plaque in AD pathogenesis

Among the pathophysiological lesions identified to date, Aβ plaques are considered the principal cytopathologic hallmark of AD in accordance with the amyloid cascade hypothesis[4-8]. At the most superficial level, the amyloid cascade hypothesis holds that perturbations in Aβ peptide formation and clearance result in a clustering of Aβ peptides to form Aβ plaques, which subsequently cause neuronal death attributable to direct and indirect neurotoxic insults (Figure 1)[8-13]. These neurodegenerative changes are hypothesized to contribute to the cognitive deficits observed in AD by disrupting the function of key brain regions involved in learning and memory, including the hippocampal formation and entorhinal cortex[14]. However, although amyloid-centric hypotheses predominate in the literature, a consensus on the role played by Aβ in AD pathogenesis has yet to be reached[15]. In all likelihood, AD pathogenesis reflects a multi-step cascade that

involves neurotoxicity secondary to Aβ aggregation, NFTs, neuronal/synaptic loss, inflammation, oxidative stress, immune dysregulation, vascular disease, and additional factors[16-22]. Regardless of the mechanism, the notion of a central role for Aβ plaques in AD pathogenesis is strongly supported by studies which estimate that 5-10% of familial AD patients carry genetic mutations which favor the production of Aβ peptides predisposed to aggregation[23-25].

Surprisingly, despite the emphasis on Aβ plaque deposition in current models of AD pathogenesis, with few exceptions[26], most studies report that the distribution and density of Aβ plaques correlates poorly with the severity of cognitive impairment[27-32]. Furthermore, approximately 20-40% of non-demented adults over the age of 65 possess considerable Aβ plaque burdens on postmortem evaluation[32-38]. Lastly, insight gained from Aβ imaging suggests that while cognitive decline progresses with time, Aβ plaque load plateaus[39]. In stark contrast to predictions made by the amyloid hypothesis, such findings are more congruent with an indirect role for Aβ plaque accumulation within a multi-factorial model of AD pathogenesis[40, 41]. Notably, the discovery of neurotoxic Aβ oligomers provides a rationale for why Aβ plaque density correlates poorly with the severity of memory impairment. In this model, Aβ species exist in a dynamic equilibrium, with soluble oligomers mediating neurotoxicity at a distance from Aβ plaques[42-45]. Reports that the abundance of Aβ oligomers correlate more closely with cognitive decline compared to Aβ plaque distribution offer support for this hypothesis[30]. Additionally, because Aβ peptide extracts are capable of inducing Aβ plaque formation when injected into the brain, it is reasonable to conclude that Aβ oligomers possess the ability to seed AD pathology[46]. Thus, despite inconsistencies between Aβ plaque load and disease progression, sufficient evidence exists to support the imaging of Aβ plaques as a biomarker of AD.

PET Imaging of Aβ Plaques

Of the early diagnostic modalities proposed for AD, PET-mediated amyloid imaging has emerged as the most promising approach with respect to developing a minimally-invasive yet clinically applicable diagnostic methodology. Despite limited clinical applicability imposed by the 20-minute half-life of C^{11}, Pittsburgh Compound B (PIB) currently represents the most comprehensively studied amyloid imaging tracer. With respect to the correlation between PIB retention and Ab plaque burden in vivo, a criteria restricted review of seven primary studies (24 case reports) concluded that sufficient evidence exists to make an association between PIB retention and Aβ plaque density[47]. Consistently, AD patients exhibit 50-90% higher rates of PIB retention compared to aged-matched, cognitively normal adults in regions such as the prefrontal cortex, precuneus, and posterior cingulate[35, 36, 48-52]. In a review of fifteen studies which cumulatively included 341 AD and 651 cognitively normal subjects, the difference in PIB retention observed in AD dementia subjects and cognitively normal controls was highly significant ($p < 0.001$). Furthermore, this study estimated the diagnostic specificity of PIB to be approximately 76%[47]. Notably however, some studies indicate that PIB retention correlates poorly with memory deficits in AD patients and healthy controls[34, 37, 50, 53-57]. According to some investigators, PIB retention may correlate poorly with observed cognitive deficits due to differences in cognitive reserve between subjects[58-62]. As a prognostic biomarker, within a follow-up period ranging from eight months to three years, 38-82% of MCI patients who screened positive for amyloid pathology via PIB imaging converted to AD compared to a mere 7% convergence rate for PIB(-) MCI cohorts[63-66]. Interestingly, although the topic is less extensively examined, clinical PET studies using [^{11}C]6OH-BTA-1 (PIB), [^{18}F]FDDNP, [^{11}C] SB- 13, [^{11}C]BF-227, [^{11}C]AZD2184, [^{18}F]BAY94-9172, [^{18}F]GE067, and [^{18}F]AV45 have reported a diagnostic utility similar to that reported for PIB. Cumulatively, this trend lends additional support to the efficacy of PET-mediated imaging of Aβ plaques as a biomarker of AD.

MR Imaging of Aβ Plaques

Inspired in part by positive PET-based imaging studies, significant progress has been made in MRI-mediated detection of Aβ plaques. Two general approaches for MRI-mediated detection have been reported: i) high-field MRI and ii) Aβ-specific contrast agents. In the first approach, investigators employing optimized spin echo acquisition methods have successfully detected histologically confirmed Aβ plaques with a 50-micrometers diameter at 9.4 Tesla[67]. With respect to the second approach to MRI-mediated Aβ imaging, a number of amyloid-specific tracers compatible with MRI have been reported[68-70]. One novel tracer designed to resemble $Aβ_{40}$ peptides, a core constituent of Aβ plaques, has been labeled with gadolinium to detect Aβ plaques in conjunction with μMRI. Impressively, the numerical density of Aβ plaques as assessed by μMRI using this tracer correlates well with immune-histochemical analysis[69]. Encouragingly, using similar MRI-based strategies, some groups have semi-quantitatively assessed Aβ plaque burden in AD mouse models using 19F and 1H MRI compatible

probes[71].

Optical Imaging of Aβ Plaques

In a trend similar to that observed during the development of PET-tracers for amyloid imaging, several groups have reported fluorescent derivatives of known Aβ plaque-binding compounds (thioflavin and Congo red) to facilitate the optical imaging of AD animal models[72-75]. However, more recently, optically based amyloid imaging approaches have become increasingly focused on near-infrared (NIR) probes in an effort to enhance the resolution of deeper brain structures[76, 77]. In the first published study in which NIR technology was employed to visualize Aβ plaques in vivo, the fluorescence intensity of the Aβ-binding dye AOI987 accurately detected the presence of Aβ plaques in a mouse model of AD[78]. In a similar in vivo study, the fluorescent signal of the amyloid-binding NIR probe THK-265 demonstrated a progressive increase in proportion to increasing burdens of Aβ plaque[79]. Despite these considerable successes however, NIR fluorescence imaging of Aβ plaques remains limited by the low number of Aβ-specific probes and poor Aβ plaque-to-background contrast. Yet, many investigators believe that these obstacles can be circumvented via the development of "smart" fluorophores which, when bound to their target, fluoresce with enhanced quantum efficiency, a shifted spectrum and/or an altered lifetime[80]. In a similar effort to advance the optical imaging of AD pathology, many groups have capitalized on the intrinsic advantages of fluorescence-lifetime imaging microscopy (FLIM) to improve the optical imaging of AD biomarkers. Using the FLIM approach, investigators can detect weak fluorescent probes in an auto-fluorescent background; a scenario in which traditional measures of fluorescence intensity are not efficacious[81-83]. Given that PET-mediated amyloid imaging lacks the sensitivity to detect individual plaques, and despite its limited ability to report Aβ plaque loads more than several centimeters below the brain's surface, many investigators predict that optical-based imaging methodologies will provide a powerful and complimentary approach to Aβ imaging[84-86].

The Role of Tau in AD Pathogenesis

Despite the dominance of amyloid-centric hypotheses, many studies have concluded that the density of NFTs comprised principally of hyper-phosphorylated tau correlate better with the severity of cognitive impairment observed in AD compared to Aβ plaques[31, 32, 87-92]. Under non-pathological conditions, tau has been implicated in the organization of cytoskeletal elements, promotion of neurite outgrowth, facilitation of membrane interactions, anchoring of phosphates and kinases, and axonal transport of vesicles and organelles[93-97]. Surprisingly, many of the tau's reported functions rely upon its ability to stabilize microtubules, a faculty which is lost upon Aβ plaque-induced phosphorylation[98]. Hyper-phosphorylated tau adopts an altered conformation, relocates from axonal to somato-dendritic compartments and loses the ability to stabilize microtubules, thus resulting in a disruption of cytoskeletal integrity, defective axonal transport and memory loss in models of tauopathy[99-104]. Interestingly, the dissociation of tau from microtubules may also be the initial step toward promoting the assembly of NFTs, as increases in the soluble tau pool may promote oligomerization[105-111]. Thus, despite the ambiguity with respect to how tau phosphorylation contributes to AD pathogenesis, virtually unanimous agreement exists regarding tau's toxicity and contribution to AD neurodegeneration[112-115]. However, although considerable evidence linking tau phosphorylation to AD pathogenesis has been published, the utility of tau as a biomarker is questionable due to i) poor corroboration between Aβ plaques and tau deposition, ii) reports of neurodegeneration in tau models without NFT formation and iii) its presence in other diseases. Assuming recent reports of NFTs in the substantia nigra and locus coeruleus are not specific to AD pathology, NFT deposition follows a stepwise topographic distribution pattern that begins in the trans-entorhinal region (Braak stage I) before affecting the entorhinal cortex (Braak stage II), hippocampus, temporo-occipital gyrus (Braak stage III), temporal cortex (Braak stage IV), parietal cortex (Braak stage V) and occipital cortex (Braak stage VI)[116-119]. By comparison, the topographical pattern of Aβ deposition is markedly different, with Aβ plaques appearing first in the neo-cortex and expanding in an anterograde fashion into allocortex (phase II), diencephalic nuclei, striatum and cholinergic nuclei (phase III), brainstem nuclei (phase IV) and the cerebellum (phase V)[120]. Given the amyloid-centric focus of current AD research, this anatomical separation of NFT from Aβ plaques must be interpreted before NFT deposition can be rationalized as a biomarker of AD. Further compounding the concerns that surround tau's ability to serve as a biomarker of AD, neurodegenerative changes and cognitive deficits have been observed in the absence of NFTs[99, 100, 103, 121, 122]. In a strategy similar to that observed in Aβ plaque research, some investigators have hypothesized that pre-tangle tau species (monomers/oligomers) underlie tau-mediated dysfunction and toxicity and thus account for studies which report dissociations in NFT

density and cognitive symptoms[113, 114, 123-126]. Regardless of tau's neurotoxic mechanism of action, studies which demonstrate that only 85% of neuronal loss can be explained by NFT formation clearly imply the existence of non-NFT mechanisms which contribute to the neurodegenerative changes observed in AD[87]. Lastly, tau expression in multiple neurodegenerative diseases including: AD, frontal-temporal dementia, progressive supra-nuclear palsy, Picks disease and corticobasal degeneration may potentially limit its efficacy as an AD biomarker. However, because NFT and tau morphologies differ between disease models and the elevations of tau in AD are significantly greater than those observed in alternative dementias, the evidence supports the notion that tau retains sufficient specificity to represent a viable biomarker for AD[127, 128]. Additionally, considerable evidence has emerged which links Aβ plaque toxicity with tau hyperphosphorylation providing additional support for its use as an AD biomarker[117, 129, 130]. For example, crossing APP-overproducing mutants with tauopathy mouse models results in significant increases in NFT formation and associated tau hyper-phosphorylation[115]. Conversely, reducing tau expression in Aβ-producing mouse models protects mice from the cognitive deficits loosely associated with high Aβ burden in the brain[131]. Clinical evidence that supports a primary role for tau in AD pathogenesis is derived from measurements of total tau levels in the cerebrospinal fluid (CSF) of AD patients. Although some studies have reported that CSF tau does not change significantly overtime in cognitively impaired patients, a review of over 50 studies have estimated the sensitivity and specificity of CSF tau levels at 80-90%, with an average increase of 300% in AD patients compared to controls[132, 133]. Extrapolating the efficacy of measuring tau in the CSF to imaging, and considering the biological evidence presented, continued efforts to leverage tau as a biomarker for AD seem warranted.

PET Imaging of Tau-comprised NFTs

Although limited by its inability to distinguish signal retention secondary to Ab plaques or NFTs, FDDNP represents the most thoroughly studied PET-based reporter of tau accumulation in human and mouse models of AD[74, 134, 135]. Following the discovery of FDDNP, ^{18}F-FSB35 and ^{18}F-FP-curcumin radiotracers have been reported. However, not only do all of these radio-ligand bind to NFTs, they also bind to Aβ plaques in the brain, which substantially limits their value with respect to investigating tau's contributions to AD pathogenesis[136-138]. Despite this limitation, the ^{18}F-FDDNP signal increases with cognitive decline and mirrors the classic trajectory of tau deposition[139]. Recently, however, a series of quinolone derivatives that bind specifically to tau NFTs in both in vivo and in vitro studies have been reported[140]. Convincingly, one of those tracers, [^{18}F] THK523, exhibits low binding in the brains of transgenic mice overexpressing Aβ but which lack NFTs, thus demonstrating its selectivity for tau[141]. Most recently, retention of the newly developed NFT-tau radio-ligand [^{18}F] T807 has been shown to co-localize significantly with tau pathology and has been leveraged for in vivo imaging[142, 143]. Cumulatively, these studies indicate that tau-specific tracers are achieving the requirements of a sensitive and specific PET probe that could be used in human imaging trials.

MR Imaging of Tau-comprised NFTs

In its infancy, MR imaging of tau relied upon the observed correlation between tau deposition and hippocampal atrophy. Because both tau deposition and brain atrophy both correlate with the severity of cognitive decline in AD patients, many study groups have hypothesized and demonstrated that patterns of gray matter loss as assessed by structural MRI can serve as an approximate in vivo surrogate indicator of tau pathology[144]. Unfortunately, considerably less progress has been made towards the development and characterization of tau-specific MRI probes. Currently, FSB and [13C]BSB are among the best characterized precursors capable of serving as potential contrast agents for magnetic resonance imaging of tau in vivo[145]. More recently, some evidence has emerged that the MRI probe CR-BSA-(Gd-DTPA) may possess the ability to detect NFTs[146]. These compounds represent derivatives of the AB plaque and NFT staining dye Congo red. Therefore, of the compounds published to date, none are specific to tau-comprised NFTs. Thus, an evaluation of MR imaging of tau as a biomarker of AD awaits the discovery of tau-specific precursors for MRI probes.

Optical Imaging of Tau-comprised NFTs

Similar to the trends observed in PET and MR imaging of tau-comprised NFTs, optical-based imaging of tau has largely been limited due to the lack of tau-specific precursor molecules. Interestingly, both FSB and BSB compounds posited for MRI also emit a fluorescent signal when complexed with NFTs making them potential candidates for in vivo imaging of tau in AD models[136]. More recently, investigators have shown that fluorescent trimethinine cyanine probes can bind to NFTs with high contrast and selec-

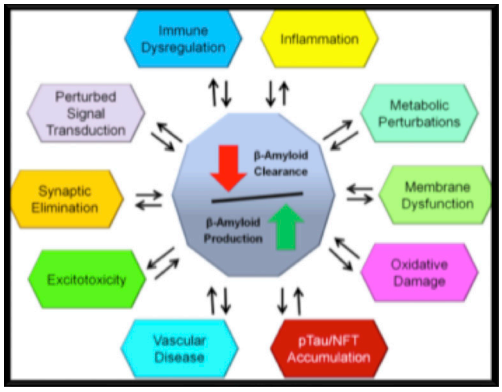

Figure 1. *Amyloid Hypothesis.* Pertubations in Aβ-peptide clearance/formation results in the deposition of Aβ plaques which initiates a multitude of downstream neurotoxic insults.

tivity over Aβ plaques[147].

When viewed in the context of an amyloid-centric perspective with respect to imaging biomarkers of AD, it is unsurprising that few precursor molecules for PET, MRI and optical-based imaging of NFTs have been thoroughly described. In addition, numerous obstacles, such as the heterogeneous nature of mature NFTs and their high degree of structural similarity with Aβ plaques, have added to the difficulty of characterizing tau-specific probes[148]. As probe development progresses, in vitro studies to determine the binding characteristics of the probe, including its equilibrium with paired helical filaments, will be needed. Notably, however, dissociation constants (K_d) and binding capacity (B max) determined in vitro are difficult to translate to in vivo conditions; thus, in vivo application in animal models of tauopathy are necessary in order to validate new probes for clinical implementation[74, 108].

Current Perspective on AD Imaging

To date, most of the progress made towards applying imaging technologies to the study of AD has been amyloid-centric. As the limitations of amyloid imaging become increasingly recognized, interest in alternative biomarkers has grown substantially. However, considering current epidemiological projections for AD, the refinement of imaging technologies for the detection of alternative biomarkers, including tau, cannot evolve over decades in a manner similar to amyloid imaging. In the years to come, high-throughput screening approaches similar to those created for Aβ plaque probe discovery must be adapted in order to expedite the validation of complimentary biomarkers for delivery into clinical trials[149]. Most likely, the imaging of AD biomarkers such as Ab plaques and tau will need to be integrated into a more holistic diagnostic panel in order to best capture the multi-factorial nature of AD.

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Dopamine: a Developmental Player in Brain Disorder Pathology

Kelli M. Money

Keywords *neurodevelopment, D1 receptor, D2 receptor, tangential migration, neuronal differentiation*

Alterations in factors that affect brain development have a long lasting impact on neuronal structure, function, or connectivity. Many neuropsychiatric disorders, such as schizophrenia and autism spectrum disorder, are associated with structural and functional alterations in frontal/prefrontal cortex and striatum, which both receive substantial dopamine (DA) innervations and express DA receptors. DA and other neurotransmitters are even expressed prior to synaptogenesis, and developmental alterations in DA and DA receptor signaling have been shown to cause enduring anatomical and behavioral alterations. However, the mechanism by which DA and its cognate receptors alter developmental processes is poorly understood.

DA and DA receptors develop in concert and are present early in development with innervation and expression patterns continuing to mature until adulthood. DAergic projections reach the rodent striatum by embryonic day (E) 14 and pass through the striatum to innervate the cortex in a lateral (E16) to medial (E19) fashion. With regards to DA receptors, there are two subgroups based on sequence, pharmacology, and G protein signaling: D1-like (D1 and D5 receptors) and D2-like (D2, D3, and D4 receptors), and all are present developmentally, appearing concurrently with DAergic innervation. D1 and D2 receptors are the most prominent throughout development in both the frontal cortex and striatum and modulate functions such as movement

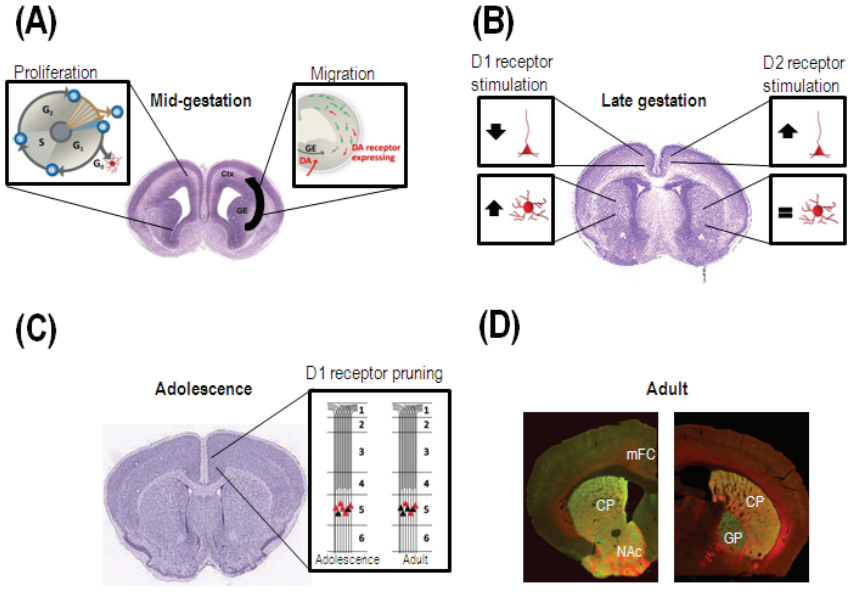

Figure 1. (A)During midgestation DA receptors have subtype-specific effects both on neuronal proliferation and interneuron migration; (B) During late-gestation DA receptors continue to regulate dendritic complexity of both cortical pyramidal neurons and striatal medium spiny projection neurons in a subtype-specific manner; (C) DA receptor expression typically peaks during adolescence and then declines. For D1 receptors in the frontal cortex, this is a "pruning" of a subpopulation of D1 receptors that are transiently expressed on the terminals of cortico-accumbens neurons; (D) Photomicrographs demonstrate the localization of D1 (red, Drd1-tdTomato reporter) and D2 (Drd2-eGFP reporter) receptors in the rostral (left) and caudal (right) striatum of the adult mouse. There is heavy labeling of D1 and D2 expressing cells within the CP and NAc with very few of these neurons co-expressing both receptors. More caudally (right) eGFP-labeled terminals can be visualized within the GP, representing the D2 receptor positive indirect pathway. D1 receptor positive axons, in contrast, bundle ventromedially to the GP and will eventually terminate in the substantia nigra and ventral tegmental area. Abbreviations: CP = caudate-putamen, Ctx = cortex, GE = ganglionic eminences, GP = globus pallidus, mFC = medial frontal cortex, and NAc = nucleus accumbens. The brain images in panels (B) and (C) are courtesy of the Allen Developing Mouse Brain Atlas and are available from: http://developingmouse.brain-map.org.

and cognition.

DA plays a significant role in neuronal proliferation, migration, and differentiation with D1 and D2 receptor activation often producing opposing effects (demonstrated in Figure 1). For example, D1-like receptor activation increases, whereas D2-like receptor activation decreases neuronal proliferation in the lateral ganglionic eminence, the basal forebrain region that produces striatal neurons. Additionally, alterations in GABAergic interneurons have been observed postmortem in neuropsychiatric disease. DA receptor activation also alters tangential migration of GABAergic interneurons from the basal forebrain to the cortex in a receptor subtype-specific manner. Disruption of dendritic and axonal growth as well as spine and synapse formation alter the quantity and nature of neural connections, which is found in neuropsychiatric diseases such as intellectual disability and schizophrenia. DA receptor activation in frontal cortex and striatum also leads to receptor subtype-specific effects in neurite outgrowth; and interestingly, D1 receptor activation also causes region-specific changes, increasing striatal and decreasing frontal cortex neurite outgrowth, that are currently not mechanistically understood. Models of DA depletion and functional excess as well as D1 and D2 receptor knockout mice show a loss of cortical and striatal dendritic spines, but moderate D1-like and D2-like receptor activation increase spine density in the striatum *in vitro*. Furthermore, DA depletion alters the number and structure of synapses and plays an important role in synapse maintenance into adulthood.

Developmental disruption of any of the above processes by DA would alter developmental trajectory and could contribute to neuropsychiatric pathology. However, understanding the signaling mechanisms required for these effects has been difficult. DA receptors are G-protein coupled receptors that couple to Gαs/olf (D1-like receptors) or Gαi/o (D2-like receptors) to either increase or decrease levels of cyclic AMP, respectively, but they can also signal through cyclic AMP-independent mechanisms like receptor heteromers and arrestin. In addition to the lack of evidence for the mechanism of the dichotomy of D1 and D2 receptor effects, studies of region-specific effects of D1 receptor activation on neurite outgrowth are superficial and have yet to provide conclusive evidence for a region-specific mechanism.

DA plays a role in typical brain development, and alterations in DAergic signaling disrupt normal developmental processes. Our understanding of the role of developmental DA perturbations in neuropsychiatric disease is still maturing and will potentially provide insight into novel treatments for the underlying pathology of neuropsychiatric disease.

Reference

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Glucagon-like Peptide 1 Receptor as a Novel Target for Drug Addiction: Preclinical Insights

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Gut hormones with effects on feeding are rapidly emerging as potent regulators of addictive behaviors through direct brain mechanisms. Glucagon-like peptide-1 (GLP-1) is one such hormone. Endogenous GLP-1 is produced both by the intestine and by a small subset of neurons in the brain. Long-lasting synthetic GLP-1 analogues have been developed to treat diabetes due to their ability to promote insulin secretion. These drugs have proven efficacy in diabetes and, interestingly, promote weight loss through brain mechanisms regulating food intake and appetite. Recent behavioral studies in rodents have suggested that central GLP-1 receptor agonism additionally promotes reductions in "addiction-like" behaviors, including hedonic feeding and alcohol context-reward associations. Here, we briefly summarize relevant preclinical studies described in a recent review1, with a particular focus on studies examining the effect of GLP-1 analogues on drug reward. We also expand upon this review through a description of mechanistic insights gained from recent publications.

Keywords *glucagon-like peptide-1; exendin-4; addiction; dopamine; reward*

Addiction to drugs is a devastating condition that adversely affects not only the addicted individual, but also families and society as a whole. Pharmacotherapeutics to supplement behavioral therapies used in treating addiction are in short supply. Promise exists, however, in a number of gut hormones with the capability to modify reward behavior in preclinical studies[2-7]. One such hormone is glucagon-like peptide 1 (GLP-1). GLP-1 is produced endogenously by intestinal L cells and possesses incretin properties in that it facilitates glucose-dependent insulin secretion from the pancreas[8]. It also potently reduces feeding and appetite, at least in part through a central effect[9-11]. Interestingly, GLP-1 is also produced by a small subset of cells located in a hindbrain region known as the nucleus of the tractus solitarius (NTS)[12]. These neurons project to a number of discrete subcortical areas[13], suggesting that GLP-1 as a neuropeptide modifies distinct signaling pathways in these regions. GLP-1 receptors (GLP-1R) are predictably also expressed in the brain[14-15].

Long-lasting synthetic GLP-1 analogues, including exenatide (Ex-4) and liraglutide, have been developed for the treatment of diabetes due to GLP-1's incretin effect. These analogues have proven useful in studying the effects of GLP-1R signaling on behavior, as they resist degradation and cross the blood brain barrier[16-17]. Their effects on feeding behavior and even food reward have been reviewed in detail elsewhere[1, 8, 18]. The focus of this review, instead, is on the effects of GLP-1 analogues on behaviors resulting from exposure to drugs of abuse, including locomotor activation and both classical and operant conditioned reward. This is presented as a follow up to a recent review published by our group and others[1]. This review will summarize its major points regarding GLP-1 analogues and chemical drug reward, and build upon its discussion of potential neurobiological substrates.

GLP-1 Analogues Reduce Reward for Alcohol, Psychostimulants, and Nicotine

Drug addiction represents a major public health concern. While behavioral therapies and public outreach have been moderately effective in approaching this problem[19], novel therapeutics are necessary to address the neurobiological mechanisms responsible for the effects of the drug and adaptations that promote or reinforce drug taking and seeking behaviors. Preclinical trials in rodents suggest that the GLP-1 analogue, Ex-4, reduces the biological effects of alcohol[20], psychostimulants[21-22] (including cocaine and amphetamine), and nicotine[23], as measured by a reduction in the open-field locomotor activation that is normally observed with acute administration of all of these drugs[24-25]. Interestingly, these drugs have distinct mechanisms of action but are all known to increase extracellular levels of the neurotransmitter, dopamine (DA)[26] (Figure 1). Thus, a parsimonious mechanism by which Ex-4 attenuates locomotor activation to these different drugs might posit that Ex-4 regulates brain DA signaling. Convincing evidence for this assertion is presented in the following section.

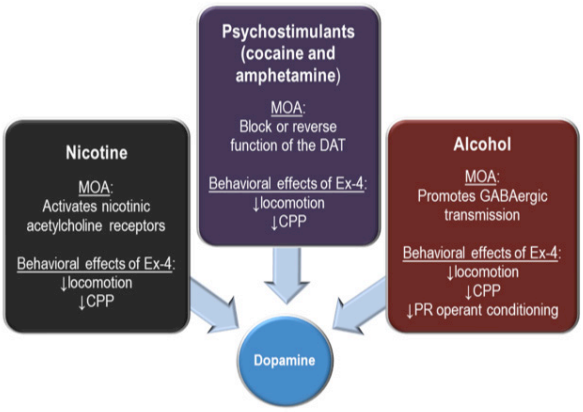

Figure 1. *Long-lasting GLP-1 analogue, Ex-4, attenuates behavioral effects of nicotine, psychostimulants, and alcohol in preclinical studies.* DAT = dopamine transporter; MOA = mechanism of action; CPP = conditioned place preference; PR = progressive ratio.

To determine whether reduced acute drug effects also result in attenuated drug reward, studies have been performed in rodents to measure the influence of Ex-4 over drug-induced classical and operant conditioned behaviors. The behavioral paradigm used to measure classical conditioning to drugs is called the conditioned place preference (CPP) test, which measures the rodent's preference (or aversion) toward an environment paired with the drug during a conditioning phase. The testing phase is performed in the absence of the drug and measures how much time the rodent spends in the drug-paired compartment versus the vehicle-paired compartment. Acute administration of Ex-4 attenuates the expression of CPP for alcohol[20,27], cocaine[2,22], amphetamine[22], and nicotine[23]. Ex-4 pretreatment prior to drug administration also reduces the acquisition of alcohol CPP[20]. Such alterations in behavior may be relevant to the observation that exposure to drug-associated contexts promotes drug seeking in rodents and potentially also human populations[28]. That Ex-4 modulates both the acquisition of CPP and its expression in these preclinical studies suggests that we consider its use in preventing the formation of drug-context associations as well as preventing context-induced relapse.

Operant conditioning in the context of drug reward refers to the association of a drug with an action that promotes acquisition of the drug. The most commonly used operant paradigm for measuring drug-motivated behavior is a progressive ratio lever press test, in which the drug becomes increasingly more difficult to obtain. In other words, the number of lever presses required to receive drug increases with progressive trials. Often, a break point, or the maximum number of times an animal will lever press to achieve a single drug administration, will be measured. Egecioglu and colleagues showed that in addition to reducing alcohol intake in a two bottle choice assay, an acute injection of Ex-4 reduces the number of alcohol rewards earned under this paradigm[20]. This finding is most simply interpreted as a reduction in the motivation to work for alcohol as it becomes harder to obtain. The authors of this study did not, however, report a break point, so this assertion cannot be made with complete certainty.

The behavioral findings reported from the studies described above are summarized in Figure 1. As a whole, they support the notion that Ex-4 modulates behavior related to acute drug effects as well as context-drug associations and drug-seeking for a diverse array of abused drugs. Still, these tests do not address all aspects of drug reward. For example, does Ex-4 reduce withdrawal symptoms? Does it prevent relapse to drug-taking in a rodent reinstatement model? These questions will need to be answered before we consider the clinical use of synthetic GLP-1 analogues for addiction. Additionally, these studies have not addressed the role of endogenous GLP-1 signaling in the behavioral response to drugs of abuse. It could reasonably be hypothesized that individual differences in endogenous GLP-1 signaling contribute to altered susceptibility to drug abuse and addiction. Studies addressing this topic will require the use of GLP-1R antagonists or GLP-1R constitutive or conditional knockout animals.

Mechanisms of GLP-1 Analogue Effect on Drug Reward: neurobiological substrates

Given that Ex-4 reduces the rewarding properties of drugs with different neural targets (receptors or transporters), we asked what mechanism most simply explains this phenomenon. DA within the mesolimbic reward system, which includes primarily projections from the midbrain ventral tegmental area (VTA) to the forebrain nucleus accumbens (NAc), is released in response to administration or consumption of alcohol, psychostimulants, and nicotine, as well as food[29]. Furthermore, DA signaling in particular brain regions is necessary for food[30] and drug seeking[31]. Alterations in DA signaling might therefore explain the reward-attenuating effects of Ex-4 for all of these drugs. Indeed, Ex-4 attenuates the accumbal DA response to alcohol[20], nicotine[23], and psychostimulants[22], as measured by microdialysis. This finding fits with the locomotor reduction observed following drug administration, which is strongly correlated with elevations in accumbal DA[24].

Several brain regions involved in producing and responding to DA are implicated in the direct actions of Ex-4. The primary mesolimbic reward areas, the VTA and NAc, have been shown to express GLP-1R as well as endogenous GLP-1 positive terminals[13]. When microinjected directly into the VTA, Ex-4 is sufficient to reduce alcohol intake over water in a two bottle choice test[27]. Ex-4 in the NAc is also sufficient to reduce feeding and food reward[32], although whether the NAc is relevant to drug reward has not been reported. Additionally, the mesolimbic reward system includes dopaminergic projections from the VTA to the amygdala[33], an important emotional processing center in the forebrain. A recent study showed that DA in the amygdala regulates food intake and food reward and that part of the anorexic effect of Ex-4 requires D2 DA receptor signaling[34]. However, they were not able to show that the D2 receptor was necessary for Ex-4-mediated reduction in sucrose reward, nor did they explore the role of the amygdala in behaviors mediated by chemical drugs. Still, this study does suggest that other dopaminoceptive areas beyond the NAc deserve attention depending on their expression of GLP-1 receptors.

While we have presented evidence that DA signaling may be central to the effects of Ex-4 on behaviors induced by different drug classes, other neurotransmitters may also be involved. Glutamate is another possible neurochemical candidate, as it has been shown to play a critical role in the learning processes underlying addiction[35, 36]. In fact, a recent study revealed that non-NMDA type glutamate receptors are necessary for the food intake-suppressive effects of Ex-4 in the VTA[37]. Since DA neurons in the VTA receive glutamatergic input from a number of regions[38], GLP-1R signaling in the VTA could influence dopaminergic neurotransmission via alterations in glutamate signaling. Somewhat surprisingly, Ex-4 bath applied to slices increases the excitability of VTA DA neurons, perhaps through a presynaptic effect[37]. This finding is unexpected given that Ex-4, at least when administered peripherally, attenuates DA elevations in the NAc[20,22-23]. However, given that the brain circuits targeted by a systemic Ex-4 injection may be very different from bath application to brain slices, these data may yet be reconciled. Future work exploring the effects of GLP-1 analogues on glutamate receptor signaling, long term potentiation or depression, and context- or cue-induced reinstatement to drugs of abuse will help shed greater light on the mechanisms by which GLP-1R signaling affects drug-elicited changes in neural function and behavior.

So far evidence suggests that Ex-4 is sufficient to reduce drug-associated behaviors when delivered peripherally or to the VTA, but it has not been reported whether any particular regions are necessary in mediating the effects of systemically administered Ex-4, which would likely be the therapeutically relevant route of administration. Furthermore, we do not even know that the GLP-1R is necessary for the effects of Ex-4 on drug reward. Addressing these issues would most benefit from targeted deletion of the GLP-1R in the brain regions mentioned here. Interestingly, while the GLP-1R is expressed in the VTA, NAc, and amygdala, it does not appear to be highly expressed relative to certain other regions[13]. This observation has led our group to hypothesize that other regions expressing very high levels of GLP-1R, such as the lateral septum, may regulate drug reward, perhaps through circuit-level influence over the mesolimbic reward system[39]. The results of targeted deletion studies will be very telling about the importance of these various regions and whether the GLP-1R is the major target in these areas.

Conclusion

GLP-1 analogues have demonstrated the ability to reduce drug effects, reward, and in some cases motivated behavior for drugs of abuse with diverse mechanisms of action. The behavioral studies reviewed herein contribute to our understanding of which drug-associated behaviors are regulated by GLP-1R signaling. However, these authors did not address all aspects of drug reward behavior. Future work will need to determine how brain GLP-1R signaling influences drug relapse, which is perhaps the most relevant aspect of addictive behavior to human therapy. It will also be important to understand GLP-1's mechanism of action in the brain in order to dissect out how it modulates particular behaviors and to develop novel therapeutics targeted to particular neuronal populations or downstream targets. As discussed here, GLP-1 as a neuropeptide likely modulates signaling by other neurotransmitters like DA and glutamate within discrete brain regions and circuits involved directly or indirectly in reward. As long-lasting synthetic GLP-1 analogues are already FDA-approved and on the market for the treatment of diabetes, barriers to translating these preclinical findings to clinical therapies are low.

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Pre-Synaptic Vesicular Pools: From Discovery to Characterization

Cassandra L. Retzlaff

Synaptic transmission is essential for nervous system function. The pre-synaptic terminal harbors synaptic vesicles that are responsible for neurotransmitter storage and release, and because of this important role, synaptic vesicles have been heavily researched for over fifty years. Studies have revealed that vesicles are of a heterogeneous population, and evidence suggests they are organized in three distinct pools. Since this description of vesicle pools, researchers have been exploring the molecular and structural differences between the individual pools. This review takes a closer look at particular methods and experiments that have been successful in elucidating how these vesicles can be defined and what separates them from each other.

Keywords *Synaptic vesicles, Endocytosis, FM dyes, Choline transporter, SynaptopHluorin*

Introduction to Synaptic Vesicles

Communication between neurons and their targets, be it other neurons or peripheral tissues, occurs at distinct sites termed synapses. These sites are important for the exchange of chemical information that can be further propagated throughout the nervous system. Dysfunction in synaptic transmission can lead to symptoms associated with a variety of diseases, such as Myasthenia Gravis, Alzheimer's Disease, and Huntington's Disease[1-2]. As neuronal communication is essential to proper nervous system function and has been implicated in such diseases, it is important to further understand synaptic processes. The synapse is composed of a pre-synaptic and post-synaptic terminal. The pre-synapse is responsible for the storage, release, and recycling of neurotransmitters via synaptic vesicles. This review will examine the beginning of vesicle research and how that work led to the discovery of heterogeneous populations of vesicles. Additionally, this review will summarize the experiments and methods that have been used to characterize these populations of vesicles and will discuss future investigations to define vesicle pools.

Discovery of Synaptic Vesicles

Investigation of the synapse by De Robertis in 1955 led to the discovery of small vesicles located in pre-synaptic terminals of neurons[3]. This finding was consistent with the earlier work of Fatt and Katz that showed neurotransmitter release was quantal, indicating a regulated release mechanism[4]. De Robertis provided a visual representation for this phenomenon. Additional evidence verifying this mechanism came from freeze-fracture experiments showing vesicle fusion with the membrane. This visual evidence was followed by inquiry of vesicle formation and maintenance[5].

Pioneering work by Heuser and Reese succeeded in showing how vesicles could be derived from the membrane of the pre-synaptic terminal. Incubating neuromuscular junction preparations with horseradish peroxidase (HRP) allowed for the engulfment of these dense molecular markers during endocytic processes. Within minutes after stimulation of the nerves, large endosomal compartments filled with HRP and could be visualized with electron microscopy (EM). These endosomal compartments then produced smaller HRP filled synaptic vesicles[5]. Fried and Blaustein conducted similar experiments in isolated central nervous system axon terminals, suggesting a common mechanism of vesicular recycling at pre-synaptic terminals throughout all of the nervous system[6-7].

Experiments involving radioactive neurotransmitters and their precursors have been used to examine transmitter synthesis, but they have also been used to uncover vesicle population differences. Experiments that employed the use of radioactive acetylcholine to fill synaptic vesicles were able to show which vesicles had undergone endocytosis. Zimmerman et al. were able to separate membrane docked vesicles and undocked vesicles, allowing a separation of radiolabeled vesicles. The sequestration of radioactivity inside vesicles was concentrated to the fraction of vesicles that were closest to the membrane[9]. These experiments gave rise to the idea that not all vesicles in the pre-synaptic terminal were the same; there was heterogeneity among the population of synaptic vesicles.

A major breakthrough in understanding these different vesicle populations came with the work of Neves and Lagnado. They quantified the endo- and exocytotic differences of vesicles us-

ing FM dyes[a] and electrophysiology techniques to observe both of these events simultaneously. The existence of three seemingly distinct vesicle pools was the main conclusion of the Neves et al. study. Short durations of electrical stimulation led to two pools of vesicles that are exocytosed and enodcytosed rapidly. Longer or stronger stimulation rates revealed a third pool of vesicles exocytosed more slowly[11] (Figure 1). These first two pools are named the readily releasable pool (RRP) and recycling pool and the final, and largest of the pools, is termed the reserve pool. Subsequent research using the three-pool model, has tried to further describe what distinguishes each pool from the other.

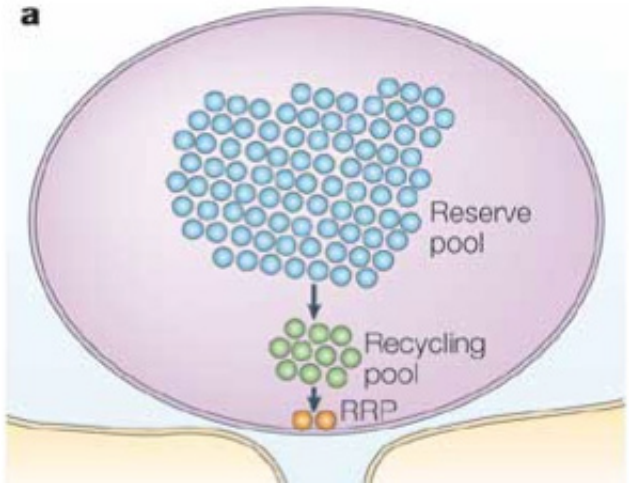

Figure 1. *Three synaptic pool model.* Reprinted with permission, Rizzoli and Betz[46].

Structure, Function, and Molecular Aspects of Vesicle Pools

Many decades of research have gone into characterizing the structural and molecular identities of the functionally defined vesicular pools.

Structure

The idea of different vesicle pools assumes that there are distinguishing characteristics of the individual groups. As mentioned above, De Robertis et al. observed vesicles in the pre-synaptic terminal, but there were no distinctive identifications separating the vesicles from each other in his images[3]. Using EM and computational reconstructions to visualize hippocampal synapses, it was observed some vesicles were organized. These studies revealed a positive linear relationship between active zone size and the number of vesicles docked. Though variable between individual synapses, these properties maintain an approximate proportional relationship to each other[12]. This work narrowed in on the readily releasable pool and how they represent the docked vesicles at the active site and how, with more space, more vesicles could be a part of this pool.

3-D Reconstruction of EM work was an important technique in analyzing vesicle organization in different parts of the terminal, but it did not address how the majority of the other vesicles were arranged in relation to each other. More recently however, advanced techniques have enabled researchers to use photo-convertible FM dyes that are visible with EM[13]. In 2012, Marra et al. used this technique to fill a subset of vesicles and then observed their spatial relationship to the membrane, as well as other vesicles. However, vesicle organization proved to be more complex than just spatial tiers of availability. The elegant reconstructions from hippocampal synapses showed that many of the vesicles were dispersed heterogeneously throughout the terminal[14] (Figure 2).

Function

Electrophysiology experiments can be used to uncover the differences in synaptic vesicle release. More specifically, by monitoring post-synaptic terminal response, kinetic changes of the pre-synaptic terminal can be determined. Pre-synaptically, capacitance measures can detect exocytotic and endocytotic events[15-16]. A more rapid recovery of resting capacitance can be seen after brief stimulations, whereas longer stimulations lead to longer times of recovery[17]. Differences in rates discovered by capacitance measures indicate multiple mechanisms of endocytosis, which could be due to differences in trafficking mechanisms[18]. Capacitance measurements have been useful in elucidating recycling rates of vesicles, but do not explain why these differences in recycling exist. Using electrophysiology in conjunction with fluorescent imaging provides structural and functional data of synaptic vesicles and produces a more complete picture of vesicle pool recycling.

The onset of a green fluorescent protein (GFP) variant that is sensitive to pH (pHluorin) allowed for the combinations of optical imaging and electrophysiology. Through genetic manipulation, neurons can express pHluorin conjugated synaptic vesicle proteins. Miesenböck et al. explored this technique and found that simultaneous post-synaptic and pre-synaptic events could be recorded[19]. pHluorin proteins fluoresce when they are in the presence of a neutral pH, such as the extra-synaptic space. However, acidification quenches the fluorescence signal upon endocytosis and the reformation of synaptic vesicles or endosomes. This process is similar to FM dyes, which are also pH sensitive. Because the fluorescence signal is attached to a

a. FM dyes are lipophilic styryl dyes that fluoresce in the acidic conditions of synaptic vesicles or endosomal compartments[10].

particular protein, it is possible to monitor subsets of vesicle populations that have differential protein expression.

Tabares et al. utilized this technique in transgenic mice expressing synaptobrevin, a synaptic vesicle protein, conjugated to a pHluorin (synaptopHluorin). Monitoring fluorescence at the neuromuscular junction (NMJ) showed distinct hot spots of release, which corresponded to areas of intense endocytosis, indicating two possible sites of these activities. Moreover, Tabares et al. was able to monitor spontaneous and evoked end plate potentials (EPPs) while observing fluorescent changes, revealing a positive correlation between the fluorescence changes (indicating exocytosis) and post-synaptic response to neurotransmitter release[20]. The work with synaptopHluorin, in conjunction with electrophysiology techniques, also proved informative about the release differences that are seen with various stimulation paradigms. Additionally, the information regarding vesicle release can be combined with recycling data of the pre-synaptic terminal, which is vital to achieve an overall picture of synaptic vesicle function. However, the question of how the vesicles themselves, on a protein level, are different still remains. Researchers interested in this question have sought to characterize vesicle pools based on molecular characteristics, such as differential protein expression.

Molecular

There are a variety of proteins that are inserted into the lipid bilayer of synaptic vesicles, interact with vesicles, and allow exo- and endocytosis of vesicles. Researchers have attempted to characterize these interactions and proteins in order to uncover the mechanism by which vesicle pools exist. Work on key proteins showing preferential interactions with individual vesicle pools are examined below.

Cytoskeleton proteins

The probability for release was what initially defined different vesicular pools. Actin's role in mobilization in most cells makes it a candidate as potentially limiting or increasing a vesicle's release capabilities. Work by Sankaranarayanan et al. found that actin's contribution within the pre-synaptic terminal is one of scaffolding and not vesicle mobilization. Using FM dyes and pharmacological intervention, Sankaranarayanan et al. determined that elimination of activity dependent dynamics of actin did not change the recycling pattern of synaptic vesicles. It was observed, however, that by disrupting actin, synapsin no longer localized to the correct place[21]. These results indicated that synapsin's role in the pre-synaptic terminal may be important with regard to vesicle organization.

Synapsin proteins are cytoskeletal proteins that have been shown to interact with synaptic vesicles, as well as with actin[21-23]. Synapsin is localized within the pre-synaptic terminal, and more precisely located distally from the plasma membrane as shown by De Camilli et al. and Hirokawa et al.[24-25]. Location of synapsin away from the plasma membrane could indicate that its interactions with vesicles are limited to only those that are not docked or primed on the plasma membrane. Characterization of the synapsin 1 knockout mouse (KO) showed few overall changes in phenotype, but structural changes of the synapses were observed using EM[26]. Fewer synaptic vesicles were observed in the synapsin 1 KO, but an even more dramatic loss was seen with two isoforms of synapsin knocked out[26-27]. In 2012, Orenbuch et al. showed that synapsin interacts with reserve pool vesicles more than with the recycling pool, and acts to immobilize the reserve pool[28]. Research on synapsin's and actin's interaction with synaptic vesicles and each other shows that cytoskeleton interactions have very particular roles within subsets of vesicles or with a specific pool, especially in regards to synapsin. These differential interactions with cytoskeletal proteins may be due to proteins that are expressed on the surface of the vesicles, which could vary from pool to pool.

Vesicular proteins

In addition to cytoskeleton proteins, vesicular proteins embedded in the membrane of vesicles likely contribute to different vesicle pools. There are proteins that are common to all vesicles (e.g. vesicular transporters and fusion machinery), but one hypothesis predicts that some proteins only exist on vesicles in a particular pool to define the pool's functional role. Investigation of the vesicle associated mem-

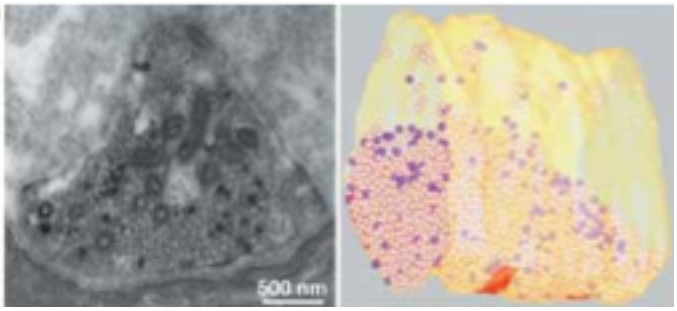

Figure 2. *Recycling pool visualization.* EM micrograph (left) and 3D reconstruction (right) of frog motor nerve terminal. The recycling pool (purple on right) is intermixed in the terminal. Reprinted with permission from Rizzoli and Betz[46].

brane protein 7 (VAMP7) has shown to only reside on a subset of vesicles. This protein is a member of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, which is involved in fusion to the plasma membrane for neurotransmitter release[29]. Robert Edwards and colleagues showed VAMP7 associated with vesicles in the pre-synaptic terminal, but also that VAMP7 positive (VAMP7+) vesicles hold different properties compared to vesicles expressing VAMP2, a distinct VAMP isoform. VAMP7+ vesicles were shown to endocytose at different rates when compared to VAMP2+ vesicles[30]. The VAMP isoform differences seen by Edwards et al. emphasizes the subtle differences between vesicle pools and suggests that particular proteins can dictate vesicle pool properties.

Molecular differences not only in fusion proteins, as seen with the VAMP7 work, but also in endocytic interacting proteins could be the key in elucidating molecular differences in vesicular pools. As described before with Heuser and Reese's work, endocytosis plays a crucial role in synaptic vesicle recycling and in maintaining the vesicle population[5]. Clathrin-mediated endocytosis is the main form of endocytosis in the pre-synaptic terminal[31]. This highly regulated process requires many proteins to effectively endocytose the proper regions of membrane and proteins to form synaptic vesicles. The clathrin adaptor proteins (APs) are able to find the appropriate cargo (i.e. proteins) through endocytic motifs, like tyrosine motifs[b] or dileucine motifs[c] that are present on cytosolic domains of proteins. APs also possess different trafficking patterns from the plasma membrane[34-36]. This confers nicely with previously mentioned evidence of the different recovery rates of vesicles. AP-2 has been shown to mediate direct synaptic vesicle reformation from the plasma membrane, whereas AP-3 first forms endosomal compartments, then vesicles bud off from those endosomes. Potential patterns of trafficking, indicated by the specific adaptor protein interaction, could dictate the vesicle pools and identities of vesicles.

Studies with the vesicular glutamate transporter (VGluT) have shown interesting results using vesicular proteins and endocytic processes that further elucidate discrepancies between different kinds of vesicle pools. There are three main isotopes of VGluT (1-3) that have complementary expression throughout the brain, and different release kinetics[37]. Voglmaier et al. hypothesized endocytic trafficking differences between the isoforms was responsible for these observed kinetic differences. By examining cytosolic amino acid sequences, it was found that VGluT1 contains two polyproline (PP) motifs, which VGluT2 and VGluT3 lack. PP motifs can interact with endophilins, which are important to clathrin-mediated endocytosis because of their role in facilitating membrane curvature[38]. The PP motif also requires a dileucine endocytic motif to be fully functional and traffic VGluT-positive vesicles properly. As mentioned earlier, dileucine motifs can be vital for providing proper AP interactions. The fact that the isoforms of VGluTs have different motifs and therefore different trafficking patterns brings an interesting new line of research to the forefront. Proteins on the vesicles, as well as the motifs they harbor, are important for distinguishing the different vesicle pools[18].

Choline Transporter's Role in Vesicular Pool Trafficking

Studies on other vesicular proteins may lead to further insights regarding the differentiation of vesicular pools in distinct neuronal populations. One such protein within cholinergic neurons is the high-affinity choline transporter (CHT). CHT is responsible for transporting choline from the synaptic space after acetylcholine metabolism back into the presynaptic terminal, so it can be used to resynthesize acetylcholine[8]. CHT and its role in choline replenishment is seen as the rate-limiting step in acetylcholine synthesis[39-42]. Most neurotransmitter transporters (e.g. dopamine transporter, serotonin transporter, etc.) are largely localized at the surface of the pre-synaptic terminal, but CHT is mainly located intracellularly on synaptic vesicles or endosomal compartments[40]. Conclusions from immuno-depletion experiments show all vesicles contain the vesicular acetylcholine transporter (VAchT), but only about half of the vesicles actually contain CHT[40]. This novel finding indicates CHT could be part of defining the heterogeneity of vesicles within cholinergic neurons.

Additionally, the localization and endocytic trafficking of CHT could suggest inherent differences about this transporter that allows for its presence on only a subset of vesicles. Further research of CHT has led to the discovery of a dileucine-like motif present in the C-terminal tail of the transporter[43]. Discussed earlier, dileucine motifs are AP binding sites for clathrin-mediated endocytosis. Further understanding of CHT's endocytic trafficking based on AP interactions may delineate what separates CHT-positive and CHT-negative vesicles from each other. Work by Misawa et al. has already shown interaction between AP-3 and CHT[44]. Continued exploration into this interaction could be beneficial in further understanding vesicle pool formation in cholinergic neurons. Studies from VGluT and CHT allow for new directions of vesicle identity research. Examination of vesicular proteins and the interactions they make could be

b. The sequence typically associated with a tyrosine motif is Yxxϕ (where Y denotes tyrosine, x a polar residue, and ϕ a large hydrophobic side chain)[32].

c. A dileucine motif amino acid sequence is KVNEQSPLLHN[33]. Many times there are deviations, but typically acidic residues will precede the leucine portion of the sequence.

the final step in elucidating the differences among the pools of vesicles in the pre-synaptic terminal.

Conclusion

Synaptic vesicles package, store, and release neurotransmitters from the pre-synaptic terminal. All of these functions are essential for proper neuronal signaling in the nervous system. Research over the past half century has been dedicated to examining these vesicles more closely to elucidate their functions and organizations. Early studies revealed the existence of synaptic vesicles, which could explain the quantal release phenomenon first observed by Fatt and Katz[4,5,45]. Further research into vesicle recycling and exocytosis led to the observation that vesicles were actually part of three distinct pools[11,46]. Characterization of these pools, by structural or molecular methods, defined distinct roles by proteins of the pre-synaptic terminal[18,28,30]. These elegant experiments have been useful and informative, but still many questions are left unanswered. Future studies on CHT could further define a mechanism of vesicle pool organization and sorting within cholinergic neurons.

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The Hippocampus Is Affected in Schizophrenia

Pratik Talati

Schizophrenia affects 1% of the general population and is characterized by positive symptoms, negative symptoms, and cognitive deficits. Within the last 20 years, the hippocampus has emerged as an intense area of investigation. In this review, the structural and functional changes seen in chronic schizophrenia, first-episode psychosis, and ultra-high-risk individuals are reviewed. These results are provided in the context of emerging literature that has focused on a sector-dependent pathology in the hippocampus. Finally, these findings are placed into a perspective of the hippocampus interacting with other brain regions to illustrate how hippocampal dysfunction can lead to a larger circuit abnormality that affects executive function and long-term memory.

Keywords *hippocampus, psychotic disorders, MRI, dopamine, GABAergic interneurons*

Schizophrenia has a lifetime prevalence of approximately 1% in the general population[1]. It usually presents in early adulthood[2] and is characterized by positive symptoms including delusions and hallucinations, negative symptoms including anhedonia[a] and avolition[b], and cognitive symptoms including memory deficits and social impairment. Schizophrenia is defined on a temporal continuum with schizophreniform disorder[c], which usually converts into schizophrenia. These illnesses have been termed "chronic disorders of the young"[3] and are projected to be a significant health burden in industrialized and rapidly industrialized regions when measured via disability-adjusted life years[4]. Even though schizophrenia has been well documented in the literature for over a century[5-6], it was not until 1985 that the hippocampus, a medial temporal lobe structure, emerged as an area significantly affected in the disease.

Structural changes of the hippocampus in schizophrenia

In a landmark study in 1985, Bogerts examined post-mortem limbic structures in healthy controls and patients with schizophrenia. The morphometric study illustrated reduced hippocampal volume in schizophrenia[7]. Using non-invasive magnetic resonance imaging (MRI) techniques, researchers have characterized *in vivo* structural alterations in individuals with chronic schizophrenia including enlarged lateral ventricles and reduced medial temporal lobe volume[8-9], most notably involving the hippocampus[8,10,11]. Meta-analyses have supported these findings by consistently reporting reductions in hippocampal volume in schizophrenia[12-13]. However, many of the studies include patients who have been treated with antipsychotic medications, and it is difficult to determine whether the finding of reduced hippocampal volume is secondary to the illness or its treatment.

Several groups aimed to determine whether the hippocampal volume changes seen in chronic schizophrenia are present early in the illness during the first psychotic break ('first-episode psychosis') or in individuals at a high risk of converting to a psychotic illness ('ultra-high-risk group'). The literature on hippocampal volume in first-episode psychosis is mixed. Some report reduced volume[14-15], while others do not[16-18]. Two meta-analyses both illustrate reduced hippocampal volume in first-episode psychosis[19-20]. However, the authors caution that the studies in the meta-analysis have potential confounds, including possible medication effect and different duration of undiagnosed illness. These can be serious confounders if the changes occur in close temporal proximity to the onset of the illness. In an effort to better understand hippocampal volume changes, some research groups have investigated individuals before they present with psychosis. Through the use of clinical assessment tools, these individuals are categorized as being in an ultra-high-risk group and have about a 30% risk of becoming psychotic[21]. In a longitudinal study examining structural changes in ultra-high-risk individuals who eventually develop psychosis and group-matched healthy individuals, notable changes were evident in many areas of the brain, including bilateral cingulate gyri and left parahippocampal gyrus[21]. This study highlights

a. Anhedonia: inability to experience pleasure from activities normally found to be pleasurable

b. Avolition: lack of motivation or drive to pursue meaningful goals

c. Schizophreniform disorder: mental disorder diagnosed when the symptoms of schizophrenia are present for at least one month but less than six months

that volume changes occur when individuals first convert to psychosis from an ultra-high-risk group. However, due to the small sample size, varying scan interval, and medication effect, no definite conclusions can be drawn about hippocampal volume changes early in the disease process. Much research is still needed to determine when these structural changes occur in the course of the illness.

There is emerging literature that attempts to better understand the location of the volume changes within the hippocampus. Before reviewing the literature on this topic, a brief review of hippocampal anatomy is necessary. The longitudinal axis of the hippocampus can be divided into anterior and posterior parts. The anterior hippocampus contains the uncus, which is defined as a folding over and rotation of the anterior portion of the hippocampus that occurs during brain development[22]. Thus, the anterior hippocampus is defined as containing more than one cut through the hippocampus (due to the uncus) in the coronal view[23]. From the lamellar hypothesis of hippocampal organization, the hippocampus can also be viewed as a series of independent, transverse slices stacked along the longitudinal axis[24]. Each transverse slice of the hippocampus contains several sectors, including the subiculum, cornu Ammonis 1 (CA1), CA2, CA3, CA4, and the dentate gyrus. These regions are connected to the entorhinal cortex through an intrinsic system called the tri-synaptic pathway (see Figure 1). The entorhinal cortex receives inputs from polymodal association cortices[25] and sends excitatory, glutamatergic projection fibers via pyramidal cells to the hippocampus and dentate gyrus. The glutamatergic fibers either project directly to the CA1 sector of the hippocampus (direct pathway) or project indirectly to the CA1 sector by traveling through the dentate gyrus and CA3 sector (indirect pathway)[26]. Fibers from the CA1 sector then project to the subiculum and other brain regions. Together, the transverse and longitudinal axes can allow for the hippocampus to be examined through a three-dimensional coordinate system (e.g., left anterior CA1, right posterior subiculum), especially in the context of disease states such as schizophrenia.

Further investigation into specific regions within the hippocampus in schizophrenia has been revealing. In a cross-sectional study investigating ultra-high-risk individuals, first-episode psychosis, and chronic schizophrenia, Velakoulis et al. found that patients with chronic schizophrenia have bilaterally reduced hippocampal volume while patients with first-episode schizophrenia have selective left hippocampal volume reduction compared to control subjects[14]. Interestingly, patients with first-episode schizophreniform disorder and the ultra-high-risk individuals did not have reduced hippocampal volume[14]. This was an important finding given that the first-episode schizophreniform group differed from the first-episode schizophrenia group only in duration of illness. The authors interpret these results to suggest that the left hippocampus is affected early in the illness, and right hippocampal volume reduction reflects illness duration. Other studies have shown that the anterior, but not the posterior hippocampus, is affected in schizophrenia[27-28]. Post-mortem studies have illustrated molecular pathology within sectors CA3, CA4, and the subiculum, with very little pathology in the CA1 sector (reviewed in [29]). Recently, shape analyses have been conducted in the hippocampus to determine sector-specific changes in schizophrenia. Studies have reported volume reductions in the anterior and midbody CA1 and CA2 subfields[30], posterior hippocampus[31], and both anterior and posterior hippocampus[32]. The results provide useful information about hippocampal shape in schizophrenia but are difficult to interpret given population heterogeneity and different shape modeling methods.

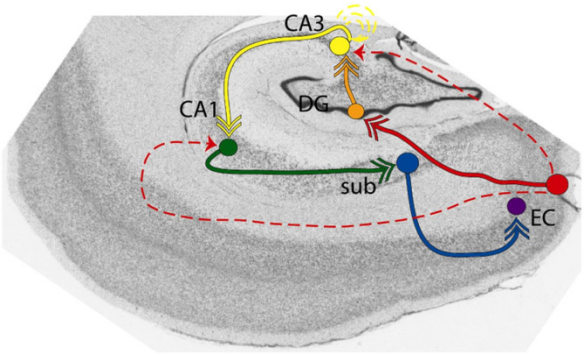

Figure 1. *Hippocampal tri-synaptic pathway.* The hippocampal tri-synaptic pathway is shown in solid lines. Dashed lines represent direct projections from the entorhinal cortex (EC) to CA3 and CA1. DG = dentate gyrus, sub = subiculum. Reprinted with permission Tamminga et al. Schizophrenia bulletin 2012.

Functional changes of the hippocampus in schizophrenia

The structural changes in the hippocampus in schizophrenia are not without functional consequences. The hippocampus is responsible for declarative memory and spatial navigation[33-34]. Meta-analyses of functional deficits in schizophrenia have illustrated significant memory impairment[35-36], especially declarative memory[37]. Further studies have shown abnormal hippocampal recruitment in schizophrenia[38-39]. In a transitive inference task, healthy controls and patients with schizophrenia had to learn a hierarchically-organized paradigm (A>B, B>C, C>D, and D>E). They were then tested on the non-relational (A>E) and the relational (B>D) pairs. Patients with schizophrenia performed

just as well as controls performed on the non-relational pair, but they were less accurate than controls on the relational pair[40]. Several studies have shown that declarative memory deficits are present in first-episode schizophrenia[41] and even before the diagnosis of schizophrenia can be made[42]. However, not all studies have shown memory deficits in the early stages of psychosis[16], highlighting the significant population heterogeneity under the diagnosis of schizophrenia.

It is important to recognize that the hippocampus is not one homogeneous functional unit. Just as the hippocampus can be partitioned into several structural units, it is currently accepted that the hippocampus is divided into several functional modules, with each subfield of the hippocampus participating in a different task[43] (see Figure 2). The entorhinal cortex functions in brief memory retention[44], and the subiculum is responsible for memory retrieval. The CA1 sector is responsible for input integration (comparing old and new stimuli); the CA3 is responsible for pattern completion (retrieving information based on partial cues)[45]; and the dentate gyrus is responsible for pattern separation (distinguishing between similar events at different time periods)[46]. These modules work together to process and route multisensory information for long-term storage in the cortex.

Given the different functions within each sector of the hippocampus, it should not be surprising that researchers have utilized high-resolution spatial imaging techniques to test hypotheses involving sector-dependent pathology in

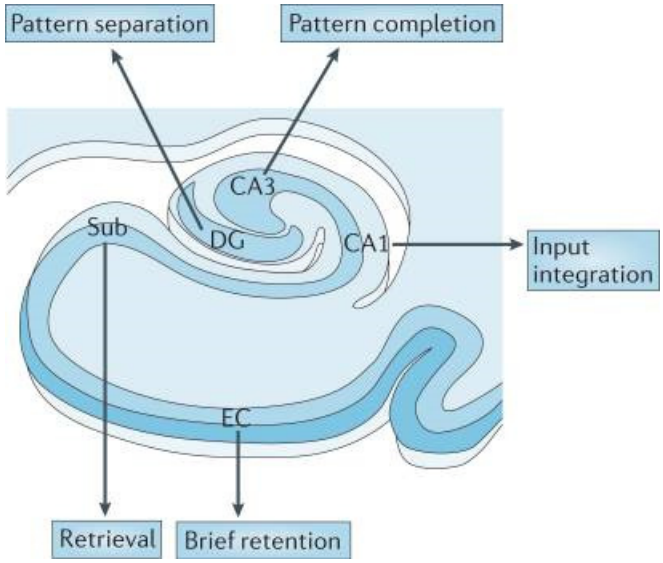

Figure 2. *Sector-specific function of the hippocampus.* The entorhinal cortex (EC) is responsible for brief retention in hippocampal-dependent memory tasks, and the subiculum (sub) is involved in retrieval of memories. The CA1 sector is responsible for input integration, the CA3 has a role in pattern completion, and the dentate gyrus (DG) is involved in pattern separation. Reprinted with permission Small et al. *Nature reviews Neuroscience* 2011.

schizophrenia. Examination of sector-dependent functional activity within the hippocampus arose out of an apparent conundrum: resting-state (baseline) positron emission tomography (PET) scans have shown increased hippocampal regional cerebral blood flow, a correlate of neuronal activity[47], while functional tasks have illustrated decreased task-dependent hippocampal activation (reviewed earlier). In order to resolve this apparent paradox, it has been recently suggested that the two are related: decreased hippocampal activation is due to increased perfusion at baseline[39,48]. Several lines of investigation support this idea, although the literature is quite mixed on the individual sectors. One group has suggested that there is increased activity within the CA3 sector[49]. If this occurs in the context of a partial dentate gyrus failure, which would create specious associations, then CA3 hyperactivity can lead to increased pattern completion, resulting in delusions or psychosis[49]. Another group has implicated CA1 sector hyperactivity in schizophrenia. Through the use of a functional MRI (fMRI) variant that measures resting-state (baseline) cerebral blood volume (CBV), a proxy of neuronal activity, the authors were able to illustrate several key findings: CBV is increased in the anterior CA1 subfield of the hippocampus, baseline CA1 CBV differentially predicts progression to psychosis from an ultra-high-risk group, and that antipsychotic medications do not likely confound the results[50]. This finding has significant implications given the CA1 sector's function in novelty detection. Hyperactivity within the CA1 sector can lead to incorrect assessment between memories that are stored in the hippocampus and new memories that are processed through the entorhinal cortex. Since all parts of the tri-synaptic pathway need to be intact to allow the hippocampus to function properly, dysfunction in the CA1 sector can lead to memory disturbances seen in schizophrenia[39,51-52]. In a follow-up study by the same group, they found that ultra-high-risk subjects have hyperactivity in the CA1 sector, which then spreads to the subiculum after psychosis onset[53]. They further illustrate that hyperactivity of the CA1 sector predicts eventual hippocampal volume loss in that sector, signifying that hippocampal hyperactivity may serve as a functional marker that precedes volume loss[53]. This research has started a new area of investigation that will hopefully lead to a better understanding of sector-dependent pathology in schizophrenia.

Functional implications for other brain regions

Anatomical studies in rats, cats, and non-human primates unequivocally show that the hippocampus is connected to many other brain structures. The connectivity of the hippocampus is quite distinctive, with the anterior hippocampus having a different connectivity profile than the

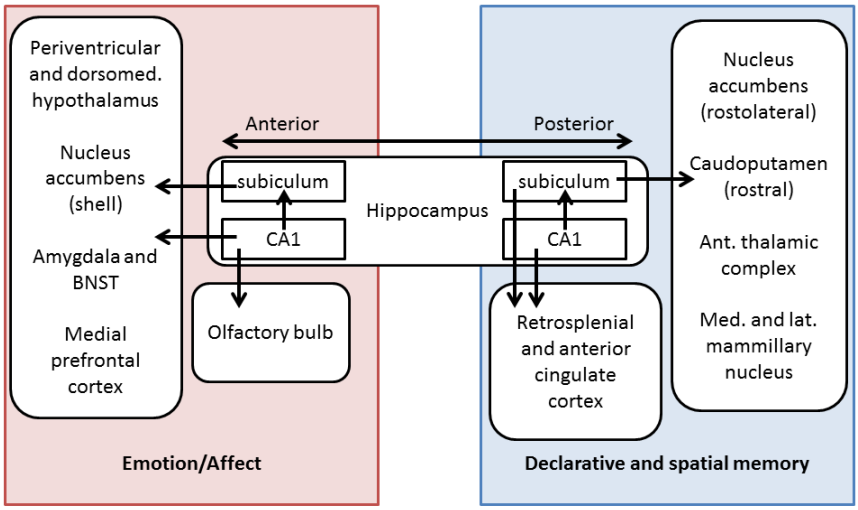

Figure 3. *Major projections from the hippocampus to other brain regions.* The anterior and posterior hippocampus have different connectivity patterns. The anterior hippocampus is believed to regulate mood and affect while the posterior hippocampus is involved in spatial memory and navigation. BNST = bed nucleus of the stria terminalis

posterior hippocampus (see Figure 3). Here the focus of the differential connectivity will be on the major outputs of the hippocampus: the CA1 sector and the subiculum. The anterior (ventral in mice) CA1 sector projects to the olfactory bulb; the anterior CA1 and subiculum both project directly to the periventricular and dorsomedial zones of the hypothalamus, the caudomedial portion (shell) of the nucleus accumbens, the bed nucleus of the stria terminalis, the amygdala, and the medial prefrontal cortex[54]. This connectivity profile is believed to allow the hippocampus to modulate emotion and affect. Meanwhile, the posterior (dorsal in mice) CA1 sector and subiculum send multisynaptic projections to the retrosplenial[d] and anterior cingulate cortices[54]. Furthermore, the posterior subiculum projects to the anterior thalamic complex, medial and lateral mammillary nuclei, the rostolateral portion of the nucleus accumbens, and the rostral caudoputamen in mice[54]. The connectivity of the posterior hippocampus to other brain regions facilitates cognitive processes such as spatial memory, navigation, and exploration[54].

The differential connectivity profile of the hippocampus may have some significance in schizophrenia. Although it is unclear which brain region is the first to be involved in the disease, the structural and functional changes that occur in the hippocampus in first-episode patients suggests that the hippocampus is at least one of the primary affected regions. A couple of recent studies have shown how changes in the CA1 sector correlate with positive symptoms in schizophrenia[50,55], illustrating the possibility that these symptoms could be due to downstream consequences (functional and eventually structural) in many different areas of the brain, including the amygdala, medial prefrontal cortex, and orbitofrontal cortex. Mechanistically, in rats, excess glutamate from pyramidal neurons has been shown to stimulate the nucleus accumbens to inhibit the ventral pallidum, resulting in a loss of inhibition to the ventral tegmental area (VTA)[56-58]. Decreased inhibition to the VTA leads to dysregulated dopamine release into several locations, including the hippocampus and prefrontal cortex; this may manifest as positive and negative symptoms of psychosis[59]. Examination of the white matter tracts in the brain using diffusion tensor imaging has suggested abnormal connectivity between frontal and temporal lobes, mainly in the uncinate fasciculus, cingulum bundle, and the arcuate fasciculus[60]. Using fMRI to examine functional circuits involving the hippocampus, researchers have shown that patients with schizophrenia have either non-optimal activation or recruit other brain regions in tasks that probe executive function and memory. For example, in a working memory and long-term memory task using nonverbal and verbal stimuli, impaired activation of the right dorsolateral prefrontal cortex and the medial temporal lobe was found in both tasks in schizophrenia[61]. In another study that investigated error monitoring, healthy controls activated the anterior cingulate, the right medial frontal, and the left posterior parietal cortex while there was no error-related increase in brain activity in patients with schizophrenia[62]. This suggests reduced error sensitivity in schizophrenia. Thus there are structural and functional abnormalities between the hippocampus and other brain regions connected to the hippocampus, although the use of antipsychotic medication remains a significant caveat. Longitudinal studies that chart progression from the early stages of psychosis into chronic schizophrenia will shed light upon the timeline of structural and functional changes in the hippocampus and other brain regions.

d. Retrosplenial region: part of the cingulate cortex defined by Brodmann areas 26, 29, and 30

Conclusion

It has been over 100 years since schizophrenia has been characterized. After the initial discovery of the hippocampus as an area of dysfunction in schizophrenia, many imaging studies have investigated the structure and function of this medial temporal lobe structure. It is quite clear that the hippocampus is reduced in chronic schizophrenia. Impaired hippocampal recruitment during related functional tasks is thought to be due to increased baseline activity. In both cases, it is unclear when in the natural history of the disease these functional and volumetric changes occur in the hippocampus.

Recent advances have been made on two fronts. The first is focusing on sector-dependent pathology in the hippocampus, and the second is capturing structural and functional changes in the hippocampus in ultra-high-risk and first-episode individuals. Longitudinal studies that encompass the progress of both of these areas will be necessary to better understand the disease progression. If indeed there are functional changes preceding structural changes that can be examined using non-invasive imaging techniques, as suggested by recent literature, then future therapies may be designed to intervene early in the disease before notable structural changes occur in the hippocampus and other brain regions. This may reduce the social and economic burden associated with treating a chronic disease.

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L-Type Voltage-Gated Calcium Channels: Structure, Regulation and Functions in the Central Nervous System

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Voltage-gated calcium channels (VGCCs) mediate selective calcium (Ca2+) influx upon membrane depolarization. L-type voltage-gated calcium channels represent one family of VGCCs that play an important role in synaptic plasticity and gene regulation. The function of L-type calcium channels is tightly regulated through Ca2+-dependent inactivation and facilitation. In addition, L-type calcium channels also undergo alternative splicing and proteolysis, which further diversify the channel regulation. In the brain, L-type channels are critical in some forms of synaptic long-term potentiation/depression and mediate activity-induced gene regulation. This review will discuss the current understandings of the regulation and function of L-type calcium channels in the brain.

Keywords *voltage-gated calcium channel, calcium-dependent inactivation, calcium-dependent facilitation, alternative splicing, proteolysis, LTP, LTD*

Calcium (Ca^{2+}) ions play an important role in regulating a variety of neuronal processes[1-3]. Ca^{2+} concentrations are tightly controlled and changes can be restricted to a confined subcellular compartment to perform a specific function[4]. Subcellular Ca^{2+} levels are regulated by multiple events: extracellular influx through Ca^{2+} channels[5], release from ER[6,7], reverse transportation by Ca^{2+} pumps and exchangers[8], as well as buffering by intracellular organelles or molecules[9]. This review will focus on the voltage-gated calcium channels, which mediate extracellular Ca^{2+} influx down the electrochemical gradients upon membrane depolarization. The processes these channels regulate are as diverse as neurotransmitter release[10], repetitive firing[11], gene regulation and synaptic plasticity[12]. I will first give a brief overview of voltage-gated calcium channels (VGCCs) and then focus on one family: the L-type calcium channels (LTCCs), which play an important role in synaptic plasticity and gene regulation. I will discuss the multi-layer regulations of LTCCs and functions of LTCCs in the brain. For a more general article of VGCCs and their regulations and functions, please refer to the 2011 review by Catterall[13].

VGCC Overview: classification and molecular components

VGCC classification

Ca^{2+} channels were identified through different types of currents they mediate. There are six types of Ca^{2+} currents based on electrophysiological and pharmacological criteria[14]. Among these, L-type currents are sensitive to dihydropyridines; P/Q-type and N-type currents are sensitive to w-agatoxin and w-CTx-GVIA, respectively[5]. Table 1 is a brief summary of the six Ca^{2+} current types and their corresponding channels.

Molecular components of VGCCs

The different types of Ca^{2+} currents are determined by the pore-forming α1 subunits (Table 1). Biochemical purifications show that a channel comprises a pore-forming α1 subunit, an intracellular β subunit, and a highly-glycosylated α2 subunit which is disulfide-linked to a trans-membrane δ subunit. In skeletal muscles and cardiac myocytes, there is also a four-transmembrane γ subunit, which is not present in neuronal Ca^{2+} channels[15].

The α subunit contains four domains (I to IV), each of which has six transmembrane segments (S1 to S6). The voltage sensor locates in S4 of each domain, and the pore is formed by S6. Ten α1 subunits are divided into three families based on their structural and phylogenic relationships. The amino acid sequences are more than 70% identical within a family but less than 40% identical among families[14]. β subunits bind to the intracellular loop between do-

	a1 subunit	Locations	Functions
L-type	$Ca_v1.1$	sk. muc.	excitation-contraction coupling
	$Ca_v1.2$	neurons, car. muc, endo. cell	E-C coupling, hormone release, gene regulation
	$Ca_v1.3$	neurons, car. muc, endo. cell	hormone release, gene regulation, repetitive firing
	$Ca_v1.4$	retina	tonic neurotransmitter release
P/Q-type	$Ca_v2.1$	nerve terminals, dendrites	neurotransmitter release, Ca^{2+} transients
N-type	$Ca_v2.2$	nerve terminals, dendrites	neurotransmitter release, Ca^{2+} transients
R-type	$Ca_v2.3$	neurons	Ca^{2+}-dependent action potential
T-type	$Ca_v3.1$	sk. muc, car. muc, neuron	repetitive firing
	$Ca_v3.2$	car. muc, neurons	repetitive firing
	$Ca_v3.3$	neurons	repetitive firing

Table 1. *Ca^{2+} currents and corresponding Ca^{2+} channels.* Abbreviations: sk. muc.: skeletal muscle; car. muc.: cardiac muscle; endo. cell, endocrine cells

main I and II of α1 subunits (a-interacting domain, AID, Fig.1)[16]. This antagonizes an ER retention signal within the I-II loop and therefore promotes the surface expression of the α1 subunit[17]. In addition, β subunits also modulate the inactivation kinetics and the opening probability of the Ca^{2+} channels (see below). γ subunits seem to enhance the voltage-dependent inactivation of the channel and reduce the Ca^{2+} currents[18, 19]. α2-γ subunit has little effect in heterologous cells but is shown to be the target of antiepileptic drugs that reduce neurotransmitter release and neuronal excitability[20].

Regulation of LTCCs

LTCCs play an important role in synaptic plasticity and gene regulation. The selective influx through LTCCs is therefore tightly controlled. In this section, multiple types of LTCC regulation will be addressed: 1) calcium-dependent inactivation; 2) calcium-dependent facilitation; 3) alternative splicing and proteolysis. LTCCs also undergo slow voltage-dependent inactivation[21], which will not be discussed in detail here. It should be pointed out that, although most studies have only isolated and examined one type of regulation, different regulation types may interact with each other to generate diversified channel regulation.

Calcium-dependent inactivation

Calcium-dependent inactivation of Ca^{2+} channels was first discovered by Brehm and Eckert in Paramecium[22]. When applied a sustained depolarization, the channel displays inward Ca^{2+} currents with a rapid decrease within 10 milliseconds, a phenomenon not seen when using Sr^{2+} or Ba^{2+} as charge carrier. This inactivation can also be manifested using a double-pulse depolarization protocol; the peak amplitude of the Ca^{2+} currents elicited by the second pulse is dependent on the amplitude of Ca^{2+} currents generated by the first one[23]. Although the exact physical process underlying CDI is still not clear, we now have a clearer idea of the calcium sensor and the molecular determinants of CDI.

Specifically, the N-terminal third of the C-tail of Ca_v1 subunits contains a putative Ca^{2+} binding motif[24] (an EF hand, Fig. 1). Deletion of the entire EF hand ablates the CDI, and donation of this domain to a non-inactivation $Ca_v2.3$ channel confers CDI[24]. However, point mutations in the EF hand that should decrease the Ca^{2+} binding affinity by 10- to 1000-fold produce only a modest effect on CDI, suggesting that the EF hand is not the bona fide Ca^{2+} sensor[25]. Yue and colleagues then identified a putative IQ-like domain C-terminal to the EF hand in $Ca_v1.2$ (Fig. 1). IQ motif[a] is known to bind to Calmodulin (CaM), therefore it is possible that CaM may serve as the Ca^{2+} sensor for Ca^{2+} channels[26]. CaM has four EF hand pairs, each of which can bind one Ca^{2+} ion. Overexpression of a mutant form of CaM, which lacks Ca^{2+}-binding in all four sites, completely ablates CDI with a dominant-negative effect in HEK293 cells[26]. Consistent with this, the alanine mutant of isoleucine in the IQ-like motif also completely abolishes the CDI, suggesting a role of CaM/C-tail interaction in CDI induction[27]. The dominant-negative effect of the mutant CaM also suggests that CaM may bind to the $Ca_v1.2$ C-tail at the basal Ca^{2+} level. This was confirmed by studies using

a. IQ motif. An amino acid sequence motif that binds to calmodulin in a Ca^{2+}-independent way. The term IQ reflects the fact that the leading amino acids are isoleucine (I, or F/L/V) and glutamine (Q).

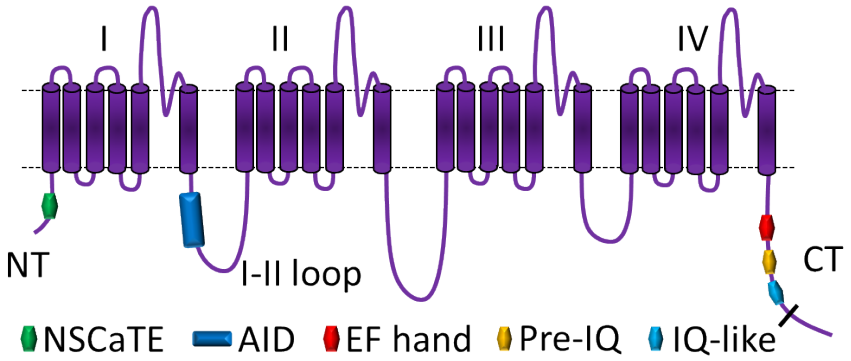

Figure 1. *Structure of $Ca_v1.2$ and $Ca_v1.3$.* The black bar C-terminal to the IQ-like domain indicates the position of alternative spliced exon 42a.

either gel shift assays or fluorescence resonant energy transfer (FRET) assays showing that the Ca^{2+}-free CaM can bind to a region that includes the IQ-like domain and a region designated pre-IQ domain (N-terminal to the IQ-like domain, Fig. 1)[28, 29].

What is the molecular machinery that blocks Ca^{2+} channels, and how is the Ca^{2+} sensor linked to this machinery? Charnet and colleagues found that coexpression of different β subunits have different effects on the inactivation of Ca^{2+} channels[30]. Since the I-II loop harbors the α-interacting domain essential for β subunit binding, they hypothesize that the I-II loop and the bound β subunit might be the pore blocker during channel inactivation. Consistent with this hypothesis, they observed an increased inactivation of the Ca^{2+} channels when overexpressing the I-II loop[30]. Studies of the voltage-dependent inactivation of Ca_v2 channels using a series of chimeras also point to the essential role of the I-II loop in channel inactivation[31]. These data suggest that the I-II loop may occlude the channel in both Ca^{2+}- and voltage-dependent inactivation. Pitt and colleagues examined the relationship between the Ca^{2+} sensor (CaM/C-tail complex) and the inactivation machinery (I-II loop), and found that: 1) the conformation of CaM/C-tail complex changes in response to Ca^{2+}, as revealed by a peak shift in gel filtration assay; 2) Ca^{2+} sensor complex binds to the I-II loop in a Ca^{2+}-dependent manner; 3) the EF hand and the multiple N-terminal regions to the IQ-like domain are required to link the Ca^{2+} sensor to the I-II loop[32]. These data from Pitt's group also explain why earlier studies found EF hand critical for CDI. Together, these data support the concept that the CaM-tethered C-tail conveys the Ca^{2+} effect to the I-II loop, which then inactivates the channel.

Another question regarding channel specificity is why LTCCs (specifically $Ca_v1.2$ and $Ca_v1.3$) exhibit local Ca^{2+} sensitivity while other VGCCs exhibit global Ca^{2+} sensitivity. This is manifested when recording the Ca^{2+} currents using Ca^{2+} buffers with different buffering kinetics. With 0.5 mM ethylene glycol tetraacetic acid (EGTA), CDI of VGCCs is present. However, applying 10mM BAPTA, which chelates Ca^{2+} much faster than EGTA and leaves only a nano-domain near the channel for free Ca^{2+}, ablates all CDIs except those of $Ca_v1.2$ and $Ca_v1.3$[33]. Recently, Yue and colleagues identified another CaM-binding domain in the N-terminus of $Ca_v1.2$ and $Ca_v1.3$[33-35]. They proposed that this element, which they termed NSCaTE (for N-terminal spatial Ca^{2+} transforming element), can confer a local Ca^{2+} sensitivity to the Ca^{2+} channels[34]. In contrast to the C-terminal CaM binding region, the NSCaTE binding to CaM is Ca^{2+}-dependent, with a higher binding affinity to the N-lobe of CaM[34]. How the local and global CDIs are coordinated and interact with each remains a key unexplored question.

Calcium-dependent facilitation

Ca^{2+} influx can also facilitate Ca^{2+} channel function. When a series of repetitive depolarization pulses are applied to the cell, Ca^{2+} current will progressively increase and plateau after five pulses[23, 36]. The Ca^{2+}/CaM-dependent Kinase II (CaMKII) has long been thought to mediate this calcium-dependent facilitation[37]. However, the mechanisms underlying facilitation of $Ca_v1.2$ and $Ca_v1.3$ appear to be different. CaMKII can directly interact with $Ca_v1.2$ (presumably through the C-tail of $Ca_v1.2$[38, 39]) and phosphorylate Ser1512 and Ser1570 sites[39]. Mutation of either site decreases facilitation of channel function, and the double mutation completely abolishes facilitation[39]. Consistently, homozygous knockin mice carrying alanine mutants of these two sites exhibit a significantly reduced CDF in cardiomyocytes[40].

Another mechanism of $Ca_v1.2$ facilitation involves

the auxiliary β2α subunit. Colbran and colleagues showed that CaMKII can phosphorylate Thr498 in the β2α subunit, and this phosphorylation event can increase the opening probability of the channel, therefore facilitating Ca^{2+} currents[41-43]. Conversely, Hofmann and colleagues found that mutation of β2α Thr498 does not affect CaMKII-mediated facilitation[39]. However, different electrophysiological protocols were used in these studies, which may not only explain the discrepancies between the results, but also point to the complex nature of the facilitation process. The Hofmann study was done with a pre-pulse protocol where a strong depolarization (+160mV, 200ms) was applied before the test pulse[39]. The Colbran study did signal channel recordings and step-depolarization[41]. It is possible that CaMKII facilitates $Ca_v1.2$ in multiple ways, each of which requires a unique phosphorylation event.

CDF of $Ca_v1.3$ may employ a different mechanism. Unlike the $Ca_v1.2$ C-tail, the $Ca_v1.3$ C-tail may not bind to CaMKII directly; coexpression of CaMKII with $Ca_v1.3$ does not facilitate Ca^{2+} influx in HEK293 cells[44]. However, when CaMKII and Densin-180[b], a CaMKII-interacting protein[45], are coexpressed, $Ca_v1.3$ channels do exhibit CDF during repetitive depolarizations[44]. Densin-180 harbors a PDZ domain that can bind to the $Ca_v1.3$ C-tail; it is possible that Densin-180 acts as a scaffold protein and tethers CaMKII to $Ca_v1.3$ channels. CaMKII may also mediate IGF-1-induced facilitation of $Ca_v1.3$[46]. Alanine mutation of Ser1486 within the C-tail of $Ca_v1.3$ blocks this CaMKII-mediated facilitation. However, it is unknown if Ser1486 is phosphorylated by CaMKII. Future studies should also investigate whether Ser1486 is critical in Densin-180/CaMKII-mediated facilitation.

The relationship between both $Ca_v1.2$ and $Ca_v1.3$ facilitation and the calcium-dependent inactivation is not well understood. Reuter and colleagues have shown that CaM is required for both CDI and CDF, and mutation of the isoleucine in the IQ-like domain can convert CDI to CDF[27]. This suggests that CDI and CDF might employ some overlapped molecular determinants, and some forms of CDF we observed might be a result of antagonized CDI. Further efforts to examine CDI and CDF side by side will help us better understand Ca^{2+} channel regulation mechanisms.

Channel regulation by alternative splicing and proteolysis

In addition to transient CDI and CDF regulation, LTCCs undergo long term and irreversible regulation by alternative mRNA splicing and proteolysis. The $Ca_v1.3$ mRNA is alternatively spliced at three regions: the I-II loop, the S2-S4 of domain IV and the C-tail[47]. The mutually exclusive splicing event in the C-tail is especially intriguing because the use of exon 42a leads to a frame shift of the downstream codons, resulting in the truncation of about 500 amino acids (Fig. 1)[47,48]. Shorter isoform Ca^{2+} channels exhibit a faster and stronger inactivation compared to the longer isoform[49].

Despite these effects of slicing on channel kinetics, the regulation of splicing events in the brain is not well understood. Soong and colleagues have generated isoform-specific antibodies to probe the spatial regulation of the two isoforms[50]. They observed a similar expression pattern of the two isoforms in the regions they examined[50]. However, whether the two isoforms show different subcellular localizations or cell-type specific localizations within a brain region and whether the splicing event is modulated in an activity-dependent manner still remains unexplored.

In addition to splice variants that generate channel diversity, the LTCC α1 subunit also undergoes proteolysis. When LTCCs were first purified in the brain, Catterall and colleagues noted the presence of different size α1 subunits[51]. Using a series of antibodies that recognize different epitopes within the α1 subunit, they showed that the α1 subunit undergoes proteolysis in the C-tail[52, 53]. The proteolysis event results in an α1 subunit that is ~35kD smaller than the full length protein. They then showed that the Ca^{2+}-dependent protease Calpain1 and Calpain2[c] cleave the α1 subunit C-tail, and that specific activation of NMDA receptor can induce the conversion of the long form of $Ca_v1.2$ into the short form[54]. This raises the possibility that LTCCs might undergo activity-dependent proteolysis that fundamentally changes their kinetics.

More recently, Dolmetsch and colleagues showed that $Ca_v1.2$ undergoes another proteolysis event that cleaves $Ca_v1.2$ in the C-tail but generates a ~75 kD protein[55]. Surprisingly, they found that this cleaved product can translocate to the nucleus and act as a transcriptional factor. Overexpression of this fragment in neurons causes expression level changes of a wide array of genes, including the sodium-calcium exchanger, cation channel TRPV4, axon guidance factor Netrin4 and RGS5, a regulator of G protein signaling[55]. Targeting of Ca^{2+} exchangers and channels suggests that there might be a transcription-based feedback mecha-

b. Densin-180. Also known as Leucine Rich Repeat Containing 7 (LRRC7), is a scaffold protein that binds to CaMKII. The PDZ domain in Densin-180 can bind to Cav1.3 C-tail.

c. Calpains. A family of Ca^{2+}-dependent protease. Calpains function at neutral pH with high substrate specificity, suggesting their role in cells is regulatory rather than digestive. Calpain1 knockout mice show deficits in LTP.

nism that regulates long term Ca^{2+} homeostasis. A more recent study has shown that the cleaved 75 kD product can inhibit promoter activity of $Ca_v1.2$, generating a negative feedback loop[56]. However, which protease mediates this proteolysis event and how this specific proteolysis event is regulated remain unknown. It is interesting that the alternative splicing literature is solely on $Ca_v1.3$, while the proteolysis literature is on $Ca_v1.2$. Whether $Ca_v1.3$ and $Ca_v1.2$ adopt different mechanisms to achieve similar channel regulation or whether two regulation mechanisms exist in both channels is also unknown.

Role of LTCCs in the Brain

LTCCs seem to play a role in both synaptic long-term potentiation (LTP) and long-term depression (LTD). LeDoux and colleagues showed that in the thalamoamygdala pathway, LTP can be induced by two different protocols: tetanic presynaptic stimulation or paired weak tetanic afferent stimulation with postsynaptic depolarization[57]. Application of NMDA receptor antagonist AVP can only block tetanus-induced LTP but not pairing-induced LTP[57]. In contrast, blocking LTCCs using Nifedipine ablates pairing-induced LTP but not tetanus-induced LTP[57, 58]. This suggests multiple forms of LTP may exist in the amygdala, and that NMDA receptors and LTCCs may contribute differently to LTP in the amygdala. However, we do not yet understand the downstream cascades that mediate these forms of LTP.

Do LTCCs also contribute to LTP in other brain areas? Johnston and colleagues showed that LTCCs may also play a role in inducing the LTP of mossy fiber input to CA3 pyramidal neurons in the hippocampus[59]. Two forms of LTP were found, and LTCC is required for the postsynaptic form of LTP[59]. Like amygdala LTP, these findings add to growing evidence that LTPs elicited by different protocols might involve different molecular and cellular mechanisms.

LTCCs also play an important role in long-term depression in striatum medium spiny neurons (MSNs). By using pharmacological blockers, researchers have realized that induction of LTD in the striatum requires functional LTCCs[60]. Which form of LTCCs is involved in LTD induction was unknown until recently, when Surmeier and colleagues found that deletion of $Ca_v1.3$ completely blocks LTD induction, while blocking the $Ca_v1.2$ with 2mM Nimodipine (which spares ~50% of $Ca_v1.3$) does not block LTD induction[61]. These findings also suggest that $Ca_v1.3$ activity is repressed by the muscarinic receptor M1, which enhances synaptic transmission, presumably by repressing $Ca_v1.3$ activity[62].

Gene expression, regulation, and protein synthesis are thought to underlie long-term memory formation. Signaling from the neuronal surface to the nucleus is critical to these processes. The phosphorylation of CREB[d] at the Ser133 site is required to activate CREB and subsequent gene expression[63]. Tsien and colleagues showed that CaM translocation from the cytosol to the nucleus can activate nuclear Ca^{2+}/CaM-dependent Kinase IV, which then phosphorylates CREB at the Ser133 site[64, 65]. Upon stimulation of the neuron, Ca^{2+} influx that causes CaM translocation is highly selective. L-type calcium channels contribute to the Ca^{2+} concentration increase to a lesser extent than N- and P/Q-type channels. However, blocking L-type calcium channels, but not N- and P/Q-type calcium channels, reduces the CaM translocation and the subsequent CREB phosphorylation, highlighting the important role of L-type calcium channels in regulating gene expression[65]. Recent data from the Tsien lab suggest that the specific mitochondria buffering effect of Ca^{2+} influx through the N- and P/Q-type calcium channels may underlie their low excitation-transcription coupling efficacy[66]. However, it still remains unclear how Ca^{2+} influx through the L-type calcium channels drives the translocation of CaM into the nucleus and activates gene transcription.

Concluding Remarks

L-type calcium channels represent an important subtype of voltage-gated calcium channels that regulate synaptic plasticity and gene expression. LTCCs are diversely regulated and critical to a variety of neuronal processes. Although we have a clear idea of some of the regulatory processes that moderate LTCC function and expression, such as calcium-dependent inactivation, other mechanisms, including calcium-dependent facilitation, splicing variation and proteolysis require more exploration, especially in the context of a specific neuronal process. A better understanding of the diverse mechanisms underlying LTCC regulation will help us better appreciate the complexity of neuronal function.

Further information: Colbran Lab URL: http://www.mc.vanderbilt.edu/root/vumc.php?site=Colbran Lab

d. CREB. cAMP response element-binding protein is a transcriptional factor that can bind to the cAMP response element (CRE) and regulate the transcription of the downstream genes.

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The Brain and Obesity

Tristan J. Watkins

Given the increasing prevalence of obesity in adults and children across the world, it is necessary to develop a greater understanding of the mechanisms contributing to weight gain and preventing weight loss. This review initially summarizes the major signaling hormones related to energy homeostasis and how their imbalance alters the hypothalamic system responsible for balancing energy intake and expenditure. Then, the role of pro-inflammatory adipokines in neural inflammation and subsequent gliosis is discussed. Finally, both established and emerging techniques for identifying inflammation associated with diet induced obesity affects global brain function, and where these effects occur within the brain, are detailed.

Keywords *obesity, neuroinflammation, hypothalamus, cytokines, histology, imaging*

Over one billion adults have a body mass index (BMI) of 25-30 kg/m^2 (overweight) worldwide and more than 300 million have a BMI greater than 30 kg/m^2 (obese)[1]. Within the last decade, the prevalence of obesity and obesity-related diseases has increased markedly within the US adult population[1]. Simply stated, body weight is determined by the balance between energy intake and energy expenditure[2]. A shift towards excess energy intake leads to increased adipose tissue deposition, obesity, and increased risk for associated metabolic disorders including dyslipidemia, cardiovascular disease, stroke, insulin resistance, and type 2 diabetes[3]. Obesity is also associated with reduced cognitive performance in adolescents and adults[4–6].

Adipose tissue, once considered to be primarily involved in energy storage, is now understood to function as an endocrine organ that secretes various bioactive substances[7-8]. Of these bioactive substances, adipose tissue secretes a variety of humoral factors, consisting of pro- and anti-inflammatory adipokines and hormonal factors[9]. Some of these hormonal factors (e.g. leptin, insulin) serve as negative adiposity feedback signals that convey information pertaining to energy storage and availability to the brain, specifically the hypothalamus[10]. The hypothalamus senses and integrates these adiposity signals and maintains energy homeostasis by controlling feeding behavior and energy expenditure[11]. Hypothalamic control of energy homeostasis, however, is not resistant to insult. Both animal models and human studies show that diet-induced obesity (DIO) and high-fat diet (HFD) can induce an inflammatory response that affects hypothalamic areas associated with control of feeding behavior and energy expenditure[12–14]. Chronic inflammation, through gliosis or insulin/leptin resistance, may result in maladaptive alterations of the hypothalamic circuitry that maintains energy homeostasis[15]. Therefore, chronic inflammation associated with HFD is one possible mechanism contributing to the increasing prevalence of obesity and the extraordinary challenges associated with weight loss[16].

Because the structural changes within the hypothalamus lead to significant functional consequences (e.g. uncontrolled weight gain), it is likely that structural changes within other neural regions will disrupt that region's typical function. Therefore, investigation of structural alterations within well-characterized regions, such as the hippocampus, frontal cortex, and striatum, may give insight into the causes of functional impairments associated with obesity (e.g. cognition, impulsivity, reward).

Major Signaling Hormones

There are three primary hormones that regulate energy intake and expenditure within the hypothalamus: insulin, leptin, and ghrelin. Insulin is synthesized and secreted by β cells in the pancreas and regulates metabolic function by acting in the liver, muscle, adipose tissue, and the brain. Systemically, insulin facilitates glucose transfer into the cell, glycogen synthesis, and glycolysis. In the brain, however, elevated levels of circulating insulin augment counter-regulatory responses to hypoglycemia and alter feeding behavior by

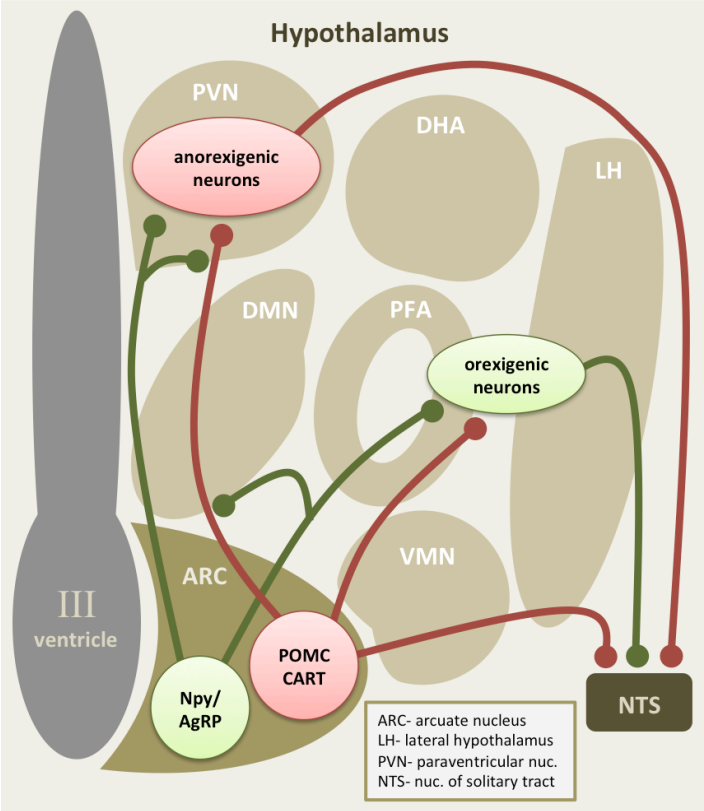

Figure 1. *Organization of hypothalamic nuclei associated with energy intake and expenditure and their projections.* POMC/CART are anorexigenic (red represents decrease feeding) and Npy/AgRP are orexigenic (green represents increase feeding). Abbreviations- (AgRP) agouti-related peptide (ARC) arcuate nucleus, (CART) cocaine- and amphetamine-related transcript (DHA) dorsal hypothalamic area, (DMN) dorsomedial nucleus, (NPY) neuropeptide Y, (NTS) nucleus of solitary tract, (LH) lateral hypothalamus, (PFA) parafornicular nucleus,(POMC) proopiomelanocortin, (PVN) paraventricular nucleus, (VMN) ventromedial nucleus

acting on insulin receptors distributed throughout the hypothalamus[17-18]. Leptin synthesis and secretion by adipose tissue is dependent upon the total amount of adipose tissue. Several rodent studies demonstrate that leptin functions as a feedback mechanism to inhibit food intake and regulate body weight by acting on leptin receptors in the hypothalamus[19–22]. Ghrelin is a peptide secreted from the stomach, gastrointestinal tract, pancreatic α cells, adrenal cortex, and the hypothalamus[23–26]. Ghrelin secretion is largely dependent upon nutritional state, showing preprandial increases and postprandial decreases[27-28]. These three primary hormones influence systemic and hypothalamic energy regulation in a highly complex manner (for a comprehensive review, see references[10,29,30]).

Hypothalamic Signaling Pathways

The hypothalamus has long been implicated as a primary region for controlling food intake and energy expenditure[31]. Lesion studies in rats first suggested the hypothalamus as a satiety center, and further studies suggest the hypothalamus also functions as a hunger center[31-32]. Stellar later posited that individual nuclei within the hypothalamus performed unique tasks[33]. Lesion studies in the ventromedial hypothalamic nucleus (VMN) resulted in hyperphagia[33]. Conversely, a lesion in the lateral hypothalamic area (LHA) resulted in hypophagia[33]. Current opinion, however, does not designate individual hypothalamic nuclei as independent centers controlling food intake. Instead, the hypothalamus is viewed as a region consisting of discrete pathways responsible for generating integrated responses to afferent input related to changes in bodily energy storage. This intricate system is highly coordinated by the arcuate nucleus (ARC).

The ARC contains neurons expressing neuropeptide Y (NPY) and agouti-related protein (AGRP) and neurons expressing pro-opiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART) that act as sensors for bodily energy stores and subsequently coordinate a complex network of neurons that ultimately control hunger and satiety signals[34]. These neurons are capable of detecting both immediate and chronic changes in levels of hormones or nutrients in the blood stream (e.g. insulin, leptin)[34]. NPY/AGRP neurons stimulate food intake when activated and are inhibited by insulin and leptin[35-36]. NPY/AGRP neurons also project to POMC/CART neurons, inhibiting their action via release of GABA[37]. POMC/CART neurons inhibit food intake and are activated by insulin and leptin[2]. Ghrelin has the reverse effect of leptin and insulin on both cell types, as it activates NPY/AGRP to initiate

feeding and inhibits POMC/CART neurons to reduce feeding[25,28,38]. Both cell subpopulations project to adjacent areas, paraventricular nucleus (PVN) and lateral hypothalamic area (LHA). The PVN is comprised of neurons that reduce food intake (anorexigenic), whereas the neuron within the LHA increase food intake (orexigenic). Signal propagated from the PVN or LHA is directed downstream to the nucleus of the solitary tract (NTS), an area implicated in satiety signaling (Figure 1)[2]. The co-localization of these distinct cell types among various nuclei suggests the hypothalamus plays a highly specialized role in energy homeostasis.

Obesity and Diet Lead to Inflammation

Accumulating evidence suggests that chronic inflammation plays a major role in the pathogenesis of obesity-related metabolic and neural dysfunction[39–41]. Beyond functioning as a long-term energy storage organ, adipose tissue plays a key role in the integration of systemic energy metabolism via secretion of various adipokines. The secretion of these adipokines is tightly controlled during normal body weight conditions, resulting in a balance of pro- and anti-inflammatory factors[42]. However, excess energy storage leads to an increase in pro-inflammatory adipokines (e.g. CRP, IL-6, IL-1β, TNFα) and a decrease in anti-inflammatory adipokines (e.g. adiponectin, SFRP5). This imbalance leads to chronic low-grade neural inflammation, insulin and leptin resistance in the brain, and increased recruitment of both microglia and astrocytes[43–46]. It has also been shown that the consumption of a high fat diet similarly affects the balance of pro- and anti-inflammatory factors, even before the development of an obese phenotype[47]. It remains unclear whether this shift towards a pro-inflammatory environment in the brain is caused by increased adipose tissue, a high fat diet, or both. There is, however, a clear understanding of which signaling cascades lead to an increase in pro-inflammatory adipokine expression.

There are a few primary signaling cascades that are consistently implicated in the neural inflammatory process. A 20-week HFD feeding study found increased reactive oxygen species production (ROS), increased prostaglandin E2 production, and upregulation of NF-κB signaling in the rat cortex[48]. In the hypothalamus, investigators reported increased activation of both Jnk and IKKβ/NF-κB pathways, as well as induction of ER stress[49–53]. Not only does activation of these pathways increase expression of pro-inflammatory adipokines, IL-1β, TNFα, and IL-6, the increased activation of these pathways has a timecourse similar to that of the development of hypothalamic insulin resistance[54-55]. Targeting these signaling cascades, specifically IKKβ/NF-κB, may be an effective strategy to reduce the chronic, low-grade neural inflammation and the insulin/leptin resistance associated with HFD and DIO.

Established and Novel Strategies for Detecting Neural Inflammation

The alterations in neural parenchyma associated with DIO and HFD have yet to be fully characterized, especially in humans. Direct, but invasive, techniques commonly used in rodent studies are not feasible in human populations. Fortunately, much work is being done to address the shortcomings associated with current non-invasive imaging techniques for human research. Within the context of neural inflammation, the currently available invasive techniques used in rodents, the non-invasive techniques utilized in humans, and effective techniques being developed for both rodents and humans will be summarized.

Invasive

Stereological techniques have been used to examine changes in hypothalamic volume and neuronal density in mice after exposure to HFD. Namavar and colleagues show that mice on HFD for 8 weeks have increased hypothalamic volumes and a decrease in hypothalamic neuron density. These results suggest two important conclusions: 1) HFD alters the neuronal structure of the hypothalamus and 2) HFD increases intercellular space in the hypothalamus, possibly by inflammation or gliosis[56]. Due to the heterogeneous population of cells within the hypothalamus (neurons, astrocytes, microglia), immunohistochemical techniques are used to examine which cell populations are affected by DIO. Using glial fibrillary acidic protein (GFAP) to examine the differential distribution of astrocytes within the hypothalamus, researchers found that DIO is associated with astrogliosis (increased astrocyte population and density) in hypothalamic nuclei proximal to the third ventricle, specifically the PVN[57]. Utilizing the histochemical microglial markers ionized calcium-binding adapter molecule 1 (Iba1) and GFAP, Thaler and colleagues observed an increase in glia cell density and morphological changes in the mediobasal hypothalamus (MBH) of rats and mice fed HFD. Importantly, these changes were observed within 1 to 3 days of HFD, persisted for up to 8 months, and were further supported by an increase in inflammatory markers in serum[13]. These studies clearly show that DIO induces changes in total number, density, and morphology of neural cells within the hypothalamus. Unfortunately, neither the techniques nor the results observed in rodent models can be directly applied to human subjects.

Magnetic Resonance Imaging

Investigators have developed novel techniques utilizing magnetic resonance imaging (MRI) to address the methodological limitations of exploring the neural changes associated with DIO in humans. This technique – building from studies correlating T2-weighted MRI signal and postmortem tissue gliosis in patients with neurodegenerative disease – quantifies subtle changes in T1- or T2-weighted signal as a marker for neural changes associated with DIO[58–60]. Simply stated, an increase in signal on a T2-weighted scan or a decrease in signal on a T1-weighted scan suggests gliosis. A landmark study by Lee and colleagues utilizing MRI and immunohistochemical techniques reported a positive correlation of T2-weighted signal in mouse MBH with BMI and a positive correlation of T2-weighted signal in mouse MBH with mean fluorescent intensity of GFAP staining in mouse MBH[14]. These results strongly support the hypothesis that neural changes associated with DIO can be measured with MRI. A similar study in humans extracted the signal from a priori regions of interest from T2-weighted MRI scans and showed a positive correlation of BMI with signal change in the MBH, suggesting that obesity is associated with MBH gliosis[13]. The results of these studies do have their limitations. As previously discussed, the MBH is a heterogeneous collection of cell types (neurons, glia) and neuron subpopulations (e.g. AGRP, POMC). Current MRI technologies do not have the spatial resolution to discern the signal from a specific cell type or neuron subpopulation. Lee and colleagues report that elevated mean fluorescent intensity of GFAP staining, but not the observed elevated Iba1 staining, correlates with T2-weighted signal. The authors then assert, citing the lack of a statistically significant correlation, that T2-weighted MRI is sensitive to changes in astrocytes rather than microglia[14]. This assertion is overreaching in that a lack of a statistically significant correlation does not translate to a lack of biological relevance. This limitation, however, does not mean that utilizing MRI in human studies is ineffective.

Positron Emission Tomography

Positron emission tomography (PET) has been used to identify brain regions with elevated levels of activated microglia in disorders associated with neuroinflammation. In Alzheimer's disease (AD), the brain demonstrates increased cytokine levels and increased concentrations of inflammatory metabolites of arachidonic acid (AA)[61–63]. Therefore, AA metabolism is likely elevated in the AD brain, particularly in areas that have high densities of senile (neuritic) plaques with activated microglia. AA cannot be synthesized de novo, converted from linoleic acid, and is unaffected by changes in regional blood flow This makes radiolabeled AA an ideal tracer for imaging brain AA metabolism, a marker for activated microglia[64–68]. Esposito and colleagues successfully used PET and radiolabeled AA in a rat model of inflammation and human AD patients to show that activated microglia can be reliably quantified[69]. A similar technique can be applied by use of [1-^{11}C] DHA (docosahexaenoic acid)[70]. There is a growing body of evidence suggesting that increased microglial activation is associated with autism spectrum disorder (ASD)[71–75]. Another way to identify activated microglia activation via PET is by use of the activated microglia radiotracer, [^{11}C](R)-(1-[2-chrorophynyl]-N-[1-methylpropyl]-3 isoquinoline carboxamide) ([^{11}C](R)-PK11195)[76–78]. Suzuki and colleagues successfully used PET and ([^{11}C](R)-PK11195) to show an increase in activated microglia in ASD patients[79]. Although these studies are focused on disorders other than obesity, they examine the common mechanism of activated microglia associated with inflammation. Future studies utilizing both PET and MRI in tandem may contribute a great deal of knowledge regarding the inflammatory effects on various brain regions associated with DIO in both rodent and human studies.

Inflammation May Affect More Than Just the Hypothalamus

The inflammatory effects associated with DIO may impact brain regions beyond the hypothalamus. Obesity, metabolic syndrome, and type 2 diabetes have been linked to various dysfunctions in cognition, impulsivity, and reward processing. Regions associated with these processes express dense populations of insulin and leptin receptors[30,80–85]. A meta-analysis performed by Thamotharan and colleagues revealed that impulsivity was greater among overweight and obese children relative to healthy weight children[86]. Elevated impulsivity in adults was also associated with increased circulating leptin levels[87]. Furthermore, adolescents with type 2 diabetes had reduced prefrontal cortex (PFC) volumes while obese adults with abnormal cholesterol profiles had abnormal white matter integrity in the PFC[88,89]. The presence of altered PFC volume and structure and increased impulsivity paired with abnormal cholesterol levels and increased levels of circulating leptin suggests that inflammation associated with DIO may play a pivotal role in neural alterations beyond the hypothalamus.

Cognitive impairment has also been documented in obese individuals. Obese adolescents have decreased arithmetic skills, spelling ability, attention, and mental flexibility[4]. The same population of adolescents also had decreased hippocampal volumes, suggesting that obesity may be altering brain regions often implicated in cognition[4]. Similar studies report a negative correlation of hippocampal

grey matter density with neuron-specific enolase, a marker for neuronal injury[90]. The striatum is also affected by DIO. Consumption of HFD impairs striatal activation of the insulin-activated signaling kinase, Akt, which leads to reduced dopamine transporter cell expression and function[91]. Disrupted dopamine homeostasis in the striatum leads to alterations in reward-driven behavior, including an increase in food intake[92–96]. Taken together, these results strengthen the hypothesis that inflammation associated with DIO has a global effect on the brain.

Conclusion

Obesity is a widespread disorder affecting children, adolescents, and adults that leads to an increase in stored adipose tissue. Adipose tissue secretes pro- and anti-inflammatory adipokines and excessive adipose tissue leads to excessive secretion of pro-inflammatory adipokines and reduced anti-inflammatory adipokines. This imbalance of pro-inflammatory adipokines results in insulin and leptin resistance, as well as gliosis. The inflammatory effects associated with DIO have been partially characterized within the hypothalamus and sparsely studied in other brain regions. There is a great deal of evidence suggesting that DIO is similarly affecting other brain regions. There are significant structural and functional deficits in the hypothalamus, hippocampus, and other brain regions associated with DIO. Rodent models have laid the framework for using non-invasive techniques to examine neural changes associated with DIO. Given the alarming prevalence of obesity and the widespread negative effects of DIO, the development and implementation of sensitive, non-invasive techniques in humans is crucial.

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