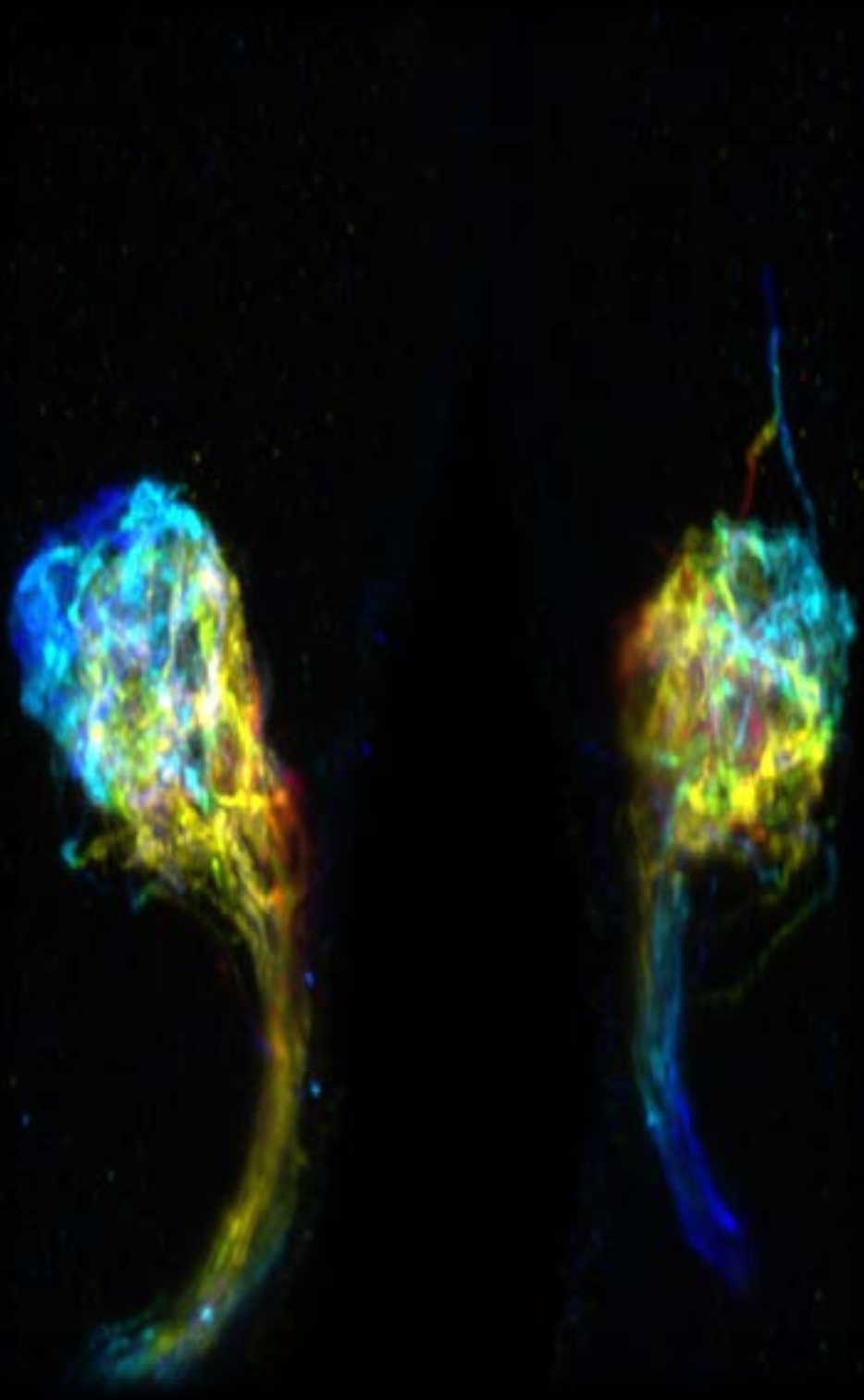
Vanderbilt Reviews Neuroscience



Volume 9 | 2017

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LETTER FROM THE EDITOR

Dear Vanderbilt Neuroscience Community,

It is my great pleasure to bring you the 2017 edition of Vanderbilt Reviews Neuroscience. This year's edition features 13 reviews written by the 2016 qualifying class covering topics ranging from the functional brain networks involved in reading comprehension to the role of serotonin signaling in mediating energy balance and the role of endocannabanoids in affective disorders. Illustrative of the successes and vigor of our students, three of the students who took their qualifying exams in 2016 have already had their qualifying exam reviews published in whole or in part in external journals. Rather than publishing those reviews here, we have included full citations to the already published versions of those reviews.

Per VRN tradition, this edition features notes from Neuroscience Student Organization President (Shilpy Dixit), the NSO Outreach Committee to update you on the activities that our students have been involved with in the past year. Additionally, the Vanderbilt Neuroscience trainees published more than 50 papers in peer-reviewed journals in the 2016-2017 academic year. We have featured summaries from a handful of these publications in the Highlights & Briefs section.

The VRN has undergone several changes – mostly behind the scenes – this year. Our editorial board is now an official Neuroscience Student Organization committee, which has greatly improved our efficacy in producing the VRN. With this change, we also convinced the VBI administration to provide a faculty mentor to assist with the publication of the VRN. Bruce Carter has graciously donated his time to be our faculty advisor and has guided us through this process. I have also worked very closely with Roz to develop a pipeline and schedule for publishing the VRN, which will serve as a template to future VRN editors. One of the most apparent changes we implemented is choosing to publish VRN online only, rather than in print. This decision allows us to publish more quickly, so that we can share the incredible work of our students more readily.

I owe a great debt of gratitude to this year's associate editors Melissa Cooper, Allyson Mallya, and Rose Follis. They have been diligent and eager in the process of communicating with students, editing reviews, and ensuring that we put together a high-quality issue. I would also like to thank Bruce Carter for his mentorship and Roz Johnson for her contributions and guidance through the publication process, all of the 2016 candidates for their contributions, and Randy Golovin for his beautiful cover art depicting the axon terminals of Drosophila Or42a expressing olfactory sensory neurons.

Happy reading,
Robin Shafer, Editor-in-Chief

MASTHEAD

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Vanderbilt Reviews Neuroscience (VRN) is open-access journal (insert link). 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 an 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 qualifications must be approved by a committe of at least four tenured or tenure-track faculty members (Phase I). All approved reviews are accepted by VRN.

2017 Editorial Board

As the oft-quoted Chinese philosopher Laozi stated, "千里之行，始於足下…. a journey of a thousand miles begins with a single step". So too does the journey for our graduate students begin as they pursue their doctoral degrees in the Vanderbilt Training Program in Neuroscience. An important step along this journey is the admission to doctoral candidacy, as the past year has witnessed another group of students that have passed their qualifying exams and have begun to focus upon their varied dissertation projects. Part of the qualifying exam process is publication of a review in a student's research area in Vanderbilt Reviews Neuroscience (VRN), which can be enjoyed within these pages. While this review represents the first publication for many of our students, it is but another step in a developing career that can be filled with long hours, exciting discoveries, new opportunities, scientific surprises, experimental setbacks, and even the occasional failure.

Now that the transition between Vanderbilt University and the Vanderbilt University Medical Center is behind us (mostly), it is time to look forward to the future of the Vanderbilt Brain Institute (VBI), the Training Program in Neuroscience and expanse of neuroscience research at our combined institutions. Part of that future is the identification of a new Director for the VBI. Regardless of who is ultimately chosen for this position however, the neuroscience research community faces both opportunities and challenges as we address significant changes in the fabric of biomedical research in the 21st century. While shortfalls in research funding have complicated the research landscape, there has never been a better time to be a neuroscientist, particularly with recent scientific discoveries and the many technological advances of the post-genomic era. Within the pages of this annual issue of VRN, our students have taken an early step upon a path in which their success will be measured not so much by their destination, but by the obstacles that they overcome while trying to succeed.

Best of luck for the coming year,

Ron Emeson

Joel G. Hardman Professor of Pharmacology, Biochemistry, Molecular Physiology & Biophysics and Psychiatry & Behavioral Sciences

A Message from the Neuroscience Program Director of Graduate Studies

Dear Readers,

This has been a year of transition, with many exciting developments and more yet to come. We had to say goodbye to several stalwarts of the neuroscience program, including Randy Blakely, who was one of the founders of the program, Karoly Mirnics, Roger Cone and, of course, our director Mark Wallace. We will miss them all, but they have all continued to rise in national stature by moving into senior leadership positions and, fortunately, Mark remains nearby as the Dean of the Graduate School. At the same time, we've gained several new faculty, including David Sweatt as the Chair of Pharmacology, and one of our longstanding program leaders, Ron Emeson, has taken the helm of the VBI as the interim director while the search for a permanent director gets underway. We are all very enthusiastic about the possibilities for the future director, especially given the commitment Vanderbilt has made to this recruitment!

We are also excited by our new crop of students. We admitted 4 new students through the direct admit route and accepted 10 from the IGP and 2 MSTPs. These students come from a variety backgrounds and locations and represent a wide range of interests. In addition to our traditional neuroscience program, our new initiatives continue to thrive, with two of the students joining the Educational Neuroscience program.

As always, our curriculum continues to evolve, with substantial input from the students. The new grant writing course (revised 8325) has received very positive reviews and the new Qualifying Exam process seems to have been well received. It continues to be a pleasure to read the outstanding reviews, written by our students as part of the exam process, published in VRN. The coming year will also see a number of changes in the curriculum, as new course directors revamp Fundamentals II (8340), Neurobiology of Disease (8365) and Methods and Experimental Design in Neuroscience Research (8352) in their own way. We are also very excited to finally have a Neuroanatomy course offered again, thanks to the recruitment of Suzana Herculano-Houzel!

Our students never cease to amaze me in their remarkable scientific accomplishments and their bold leadership. This year approximately 16 students successfully defended their PhDs, with many important and exciting discoveries that have significantly advanced the field of neuroscience. Several students have received recognition for their work, including Kathryn Unruh, who was named a Weatherstone Fellow of Autism Speaks. Our students also continue to organize our annual retreat, the Brain Blast outreach program, as well as other activities and events, including running this unique publication, which they founded. It is a privilege to serve as the Director of Graduate Studies for such a fantastic group of students!

Sincerely,

Bruce Carter

An Update from the Neuroscience President

It was truly an honor to serve as the Neuroscience Student Organization president this year. The purpose of the NSO and goals we set are focused on improving the quality of life for Neuroscience graduate students through curricular preparation, community engagement and public outreach. This year, Iliza Butera, Director of Photography enhanced the purview of the media division of the NSO and Vanderbilt Reviews Neuroscience joined the NSO. I would like to take this opportunity to welcome our new students and invite them to take advantage of all the Neuroscience community has to offer by joining and contributing to committees like these and attending events.

I would like to congratulate the new Neuroscience doctoral candidates on successful completion of the qualifying exam and on your work highlighted in this publication. I would like to congratulate the editors and contributors to Vanderbilt Reviews Neuroscience for another great publication. I also want to extend a special thank you to the Greg Salimando, Sahana Nagabhushan Kalburgi and Brandon Moore of the Academic Committee for organizing and preparing the students for this year's exams and to Allyson Mallya and Lauren Bryant of the Social Committee for giving everyone the opportunity to blow off some steam during our student socials.

This was a big year for community engagement and outreach. Thanks to the efforts of Stephen Bailey, Rose Follis and Gabriella DiCarlo of the NSO Outreach committee, Neuroscience students had the opportunity to teach classes about the brain to elementary school students in the Nashville area. For Brain Awareness Month, the NSO Outreach Committee organized a Brown Bag Neuroscience Seminar series for the Vanderbilt community and partnered with TSU for another successful Brain Blast community event. Brain Awareness Month would not have been possible without the support of the faculty outreach committee and staff. The Alzheimer's Association Congressional Ambassadors team also partnered with NSO Outreach to organize the first ever Vanderbilt Research Symposium in which faculty researchers at Vanderbilt had the opportunity to share their work with the public. I also want to thank the Nashville Public Library for inviting Neuroscience doctoral candidate Jacob Ruden and myself to share our work in Alzheimer's research with the community. Shawn Barton went above and beyond as Retreat Coordinator in organizing the 20th Annual Neuroscience Retreat at beautiful Cheekwood Botanical Gardens. The retreat is a great opportunity to welcome new faculty and students and catch up on research and achievements in our Neuroscience community and a great way to kick off the academic year.

Finally, I want to show my appreciation for the enduring commitment of the Neuroscience faculty and staff in supporting the Neuroscience students, especially during the search for a new director for the Vanderbilt Brain Institute. I would like to stress that it remains a priority for the NSO that the new director continue to support efforts to strengthen the training program. I am grateful to have served on the NSO and to have had the opportunity to work alongside so many dedicated colleagues.

Shilpy Dixit
2017 Neuroscience Student Organization President

Brain Blast | Community Outreach

Community outreach is a critical component of the Vanderbilt Brain Institute's mission. The Neuroscience Student Organization Outreach Committee (along with the Vanderbilt Brain Institute's Faculty Outreach Committee) plans and hosts a variety of seminars, lectures, and outreach events throughout the year. These events are designed to engaged the Nashville community in ongoing neuroscience research and brain health awareness. "The efforts of the VBI in outreach and engagement build enthusiasm and support for science, while promoting trust between the scientific community and the public," explains Shilpy Dixit, President of the Neuroscience Student Organization.

The VBI's largest outreach event every year is Brain Blast, which is a highlight of the annual VBI celebration of Brain Awareness Month. This event is designed to expose elementary and middle school students to neuroscience and the importance of brain health. This year, the VBI partnered with Tennessee State University to host Brain Blast on TSU's campus. Over 20 VBI-affiliated laboratories facilitated interactive booths that showcased ongoing research, demonstrated research techniques, and taught important lessons about brain structure and function. Participants extracted DNA from strawberries, visualized brain waves using EEG, dissected brains, and learned about the structure of neurons. In addition to this event, the VBI also hosted a number of community lectures by PhD students during Brain Awareness Month.

This year, we also extended our outreach efforts to include a classroom-based educational series. PhD students, postdoctoral fellows, and faculty visited Nashville Metro Public Schools to teach basic neuroanatomy and to perform hands-on brain dissections with students. Additionally, PhD students gave educational talks on Alzheimer's Disease and dementia at various Nashville public libraries. These events demonstrate the VBI's ongoing dedication to disseminating knowledge into the community and engaging the public in scientific research.

Gabriella DiCarlo

RESEARCH HIGHLIGHTS

Alcohol-abstinence-induced depression: Reversal by ketamine and MAG lipase Inhibition

Allyson Mallya, Graduate Student

Alcohol use disorder (AUD) is a complex brain disease defined as a "problematic pattern of alcohol use leading to clinically significant impairment or distress" (American Psychiatric Association, 2013). AUDs are highly prevalent, affecting an estimated 16 million people in the United States, and are strongly comorbid with mood and affective disorders, including depression and anxiety. Although each disorder can exacerbate the symptoms of the other, clinical strategies have focused on treating the diseases consecutively instead of simultaneously.

Katie Holleran, Ph.D., explored the influence of alcohol abstinence (withdrawal) on affective state while a neuroscience graduate student in the lab of Danny Winder, Ph.D., Professor of Molecular Physiology & Biophysics and Psychiatry and Director of the Vanderbilt Center for Addiction Research. In a report published in Neuropsychopharmacology, Holleran and colleagues established that prolonged but not acute alcohol (ethanol; EtOH) abstinence induces a persistent depression-like phenotype in female mice. This effect was reversible via treatment with the N-Methyl-D-aspartate receptor (NMDAR) antagonist ketamine as well as with the monoacylglycerol lipase (MAGL) inhibitor JZL-184, which boosts levels of the endocannabinoid (eCB) 2-Arachidonoylglycerol (2-AG).

The authors gave female mice, chosen in part because of the higher rate of depression in females than males, access to sippers with either 10% EtOH or water; control mice received water in both sippers. After 6 weeks mice exhibited a clear preference for the 10% EtOH, replicating previous findings.

Mice then underwent a period of forced abstinence followed by behavioral testing. In a forced swim test increased immobility relative to controls was observed in mice EtOH-abstinent for 18 days, but not for 24 hours. EtOH-abstinence for 14 days did not produce a significant effect in an elevated plus maze test for anxiety. Together, this indicates that prolonged EtOH-abstinence induces a depression- but not anxiety-like phenotype.

Holleran and colleagues then tested mice on a novelty-suppressed feeding test (NSFT), which measures latency to eat familiar food in a novel environment. Mice that were both 15 and 35 days EtOH-abstinent had higher latencies than controls, demonstrating that EtOH abstinence produces a persistent affective disturbance. Treatment with ketamine, an antidepressant effective in both humans and rodents, before NSFT testing reversed the increased latency in mice abstinent for 15 days to control levels. Another NMDAR antagonist, memantine, did not elicit this reversal.

The authors also tested eCB modulation of EtOH-induced affective behavior. Mice were treated with JZL-184 prior to NSFT testing. Similar to ketamine, JZL-184 reduced latency to baseline levels in mice abstinent for 15 days. Co-administration of JZL-184 with rimonabant, an inverse agonist for the CB1 receptor to which 2-AG binds, eliminated the ameliorating effect of JZL-184. Mass spectrometry results showed that levels of 2-AG and anandamide, another endogenous eCB, were increased in the basolateral amygdala but not in other brain areas in mice currently drinking EtOH compared to those 15 days abstinent.

Taken together the data suggests a role for protracted EtOH withdrawal in inducing an enduring affective disturbance that is reversible via two distinct pharmacological treatment pathways. This study contributes to the understanding of how alcohol abstinence influences affective state and lays the groundwork for developing novel treatment options for alcohol-withdrawal-induced mood and anxiety disorders.

Learn More: Holleran KM, Wilson HH, Fetterly TL, Bluett RJ, Centanni SW, Gilfarb RA, Rocco LE, Patel S, Winder DG. Ketamine and MAG Lipase Inhibitor-Dependent Reversal of Evolving Depressive-Like Behavior During Forced Abstinence From Alcohol Drinking. Neuropsychopharmacology 2016; 41:2062-71.

RESEARCH HIGHLIGHTS

Contralateral volumetric changes in brain regions correlate with locus of seizures in epilepsy patients

Melissa Cooper, Graduate Student

Neuronal interconnectivity is of particular importance in Temporal Lobe Epilepsy (TLE), a common form of adult epilepsy characterized by recurrent temporolimbic seizure activity. A hallmark of the disease is widespread grey matter atrophy; however, the literature contains inconsistencies regarding the pattern and extent of grey matter degeneration. In this study, Conrad et al. provide a novel whole brain assessment of volumetric correlation in TLE patients. To do so, they quantified differences in regional volumetric correlation in right (RTLE) and left (LTLE) sided TLE patients compared to healthy controls to better understand the subtle patterns of coordinated volumetric change that may be related to TLE progression.

This study demonstrated increased correlation in volumetric atrophy within regions in the contralateral hemisphere including the temporal, limbic, and subcortical areas for both RTLE and LTLE patients. Increased positive correlations among regional volumes suggests these homologues undergo mutually trophic changes with other regions in these lobes, highlighting a possible pattern of loss. These patterns of volumetric change create "networks" of regions not typically related among healthy controls. These networks have been highlighted over multiple studies, through both tracking energetic expenditure and imaging the structures themselves.

One hypothesis is that synchronous, gradual atrophy across contralateral region networks over the course of TLE causes a positive correlation in grey matter volume. Though not mutually exclusive, another intriguing possibility involves compensatory mechanisms. These mechanisms would potentially be activated in response to dysfunction in the ipsilateral epileptogenic regions. In this study, Conrad et al. additionally show that volume of the contralateral inferior temporal region relative to total grey matter volume was increased in patients compared to controls. Taken together with their findings of increased correlation among contralateral temporal, limbic, and subcortical regions, these data provide further evidence of compensatory mechanisms in which the volume of gray matter in networks contralateral to the seizure side is preserved or even increased compared to the rest of the brain due to altered functional or metabolic demands.

An important distinction to make between volumetric correlation and other measures of correlation, such as the BOLD signal, is that here each subject contributes a single value (here, regional volume) rather than a correlational value such as the correlation of BOLD signals between two regions over time. Individually, these volumes do not provide evidence of connectivity, so only as a group can one determine correlated alterations between regions. This inherently imposes a limitation for cross-sectional studies of volumetric correlations; these measures cannot be related directly to subject level clinical variables such as disease duration, seizure frequency, or measures of cognitive dysfunction. Due to their careful minimization of the effects of age, gender, head size, and MRI head coil, the authors here lacked the statistical power to create subgroups of subjects related on any of these measures. However, they suggest future studies may use a longitudinal approach to provide further evidence of either compensatory mechanisms or novel networks in TLE patients.

Learn More: Conrad, N.D., Rogers, B.P., Bassel, A., Morgan, V.L. Increased MRI volumetric correlation contralateral to seizure focus in temporal lobe epilepsy. Epilepsy Research. 2016; 126:53-61.

RESEARCH HIGHLIGHTS

Short-term Multisensory adaptation can be captured with EEG

Robin Shafer, Graduate Student

Integration of auditory and visual stimuli from a single source requires adaptation to small differences in the timing of the signals from each modality. This is because sound and light travel at different speeds, so the distance of the source of the stimuli affects the offset in the timing between the two modalities. In order for the brain to unite the two modalities and attribute them to a common source, it must rapidly adapt to changes in the synchronicity of the auditory and visual stimuli. Previous studies have demonstrated this rapid adaptation at a behavioral level, but this study is the first to identify neural mechanisms involved in this adaptation process. Simon et al. designed a simultaneity judgment task where auditory (pure tone) and visual (circle) were presented at various temporal offsets ranging from auditory leading visual by 300ms to visual leading auditory by 300ms. Participants were required to indicate via button press whether the stimuli occurred at the same time or at different times. They performed the task while wearing an EEG cap to measure electrical activity at the scalp. EEG is commonly analyzed by averaging together the time series of the electrical potential over a series of trials time locked to a specific stimulus or condition. In this study, analyzed trials according to the order in which they occurred. They grouped together the trials that followed trials in which the auditory stimulus led the visual stimulus at long and short offsets and compared the neural and behavioral responses to trials in which the visual stimulus led the auditory stimulus at long and short offsets. The results indicated that the behavioral response shifted to the paired stimuli following trials where the auditory or visual stimulus had a large lead, consistent with previous studies. The EEG responses demonstrated that for trials following trials in which the auditory or visual stimulus had a large lead,

there was a shift in the neural response occurring 125ms after the second stimulus in the pair. This shift was also present following trials where the auditory stimulus had a relatively short lead compared to the visual stimulus. These differences in the neural responses occur at latencies that are generally associated with stimulus evaluation and decision-making indicating that the synchronicity of the immediately preceding stimuli are contributing to the neural evaluation of the subsequent stimuli. This was found in trials following a broader range of auditory leading trials than trials following visually leading trials, which is likely due to the fact that it is very common in the natural world for visual stimuli to lead auditory stimuli and much more rare for auditory stimuli to lead visual stimuli. Therefore, the neural adaptation occurs more readily following less familiar stimulus conditions.

This study provides the first neural evidence that the brain rapidly modifies its response to stimuli based on the stimuli it perceived immediately prior.

Learn More: Simon, David M., Noel, Jean-Paul, & Wallace, Mark T. Event Related Potentials Index Rapid Recalibration to Audiovisual Temporal Asynchrony. Frontiers in Integrative Neuroscience. 2017

RESEARCH HIGHLIGHTS

When the (Interocular) Pressure is Too High: Astrocyte Redistribution as an Additional Stressor Contributing to Optic Nerve Damage in Glaucoma.

Rose Follis, Graduate Student

Glaucoma is the second leading cause of irreversible blindness in the world. The most common form of glaucoma is associated with increased interocular pressure (IOP) which leads to optic nerve damage and degeneration of retinal ganglion cells (RSCs). However, management of IOP along is not always able to prevent the progression of RSC degeneration and some forms of glaucoma are not associated with increased IOP, suggesting that other stressors likely contribute to optic nerve damage. In this study, Cooper et al. (2016) utilized the DBA/2J mouse model of hereditary glaucoma to explore the early pathogenesis of optic nerve damage and determine what other factors may contribute to RSC loss.

Cooper et al. (2016) found distinct age dependent changes in the morphology of the DBA/2J optic nerve preceding RSC degeneration after examining nerve cross sections. As the animals aged, the area of the optic nerve increased. The expansion resulted both from more spread out axons, or decreased axon packing density, and from increased total glial area. Moreover, the axons themselves had altered morphology, exhibiting axoplasmic swelling and disordered neurofilament accumulation. Since neuronal transport is highly depended on regulated axon diameter and cytoskeletal arrangement this suggests that RSCs undergo a period of inhibited axonal transport prior to degeneration. Interestingly, while expansion of axons was found to be age dependent, high IOP also correlated with increased axon size, suggesting both factors independently contribute to pre-degenerative axon expansion.

Additionally, while glial area tended to increase as the optic nerve swelled, astrocytic and microglia organization were heavily altered. Typically, astrocytes provide trophic and environmental support to RSCs and are dispersed throughout the optic nerve, extending processes that stretch between and around axons. However, in DBA/2J nerves, during the critical early period of axon expansion, astrocytes were found to have reduced process area were distributed aberrantly at the nerve edge. Microglia, key mediators of inflammation, were also found to be reduced in the nerve. Interestingly, as axonal expansion progress beyond the critical threshold for loss, indicating degenerative progression throughout the nerve, the glia again tended towards an even distribution from the center to the edge of the nerve. This suggests that the remodeling of glia is a feature primarily of early, possibly pre-symptomatic glaucoma pathogenesis.

Taken in its entirety, Cooper et al. (2016) suggests that glaucoma pathology may result, not just from increased IOP, but from the additional contribution of age dependent axon expansion, and decreased axons density. Additionally, since axons rely on trophic and protective support from astrocytes, their extensive retraction and rearrangement concurrent with axon expansion may constitute an additional stressor in the nerve and lead to further induction of RSC degeneration.

Learn More: Cooper, M.L., Crish, S.D., Inman, D.M., Horner, P.J., Calkins, D.J. Early astrocyte redistribution in the optic nerve precedes axonopathy in the DBA/2J mouse model of glaucoma. Exp Eye Res. 2016. 150:22-33. PMID:26646560

RESEARCH BRIEFS

Stress-resilience: A role for endocannabinoid signaling

Allyson Mallya, Graduate Student

Stress is a potent risk factor for the development of mood and affective disorders. These disorders are common, a leading cause of disability, and associated with significant social and economic burdens. Interest in understanding neurobiological mechanisms conferring stress-resilience – the active process of adapting well to various traumas or stressors – has increased, with the focus centering on the endocannabinoid (eCB) system, which has a demonstrated role in modulating stress response and anxiety.

A recent study led by Rebecca Bluett, Ph.D., and Rita Báldi, Ph.D., from the lab of Sachin Patel, M.D., Ph.D., sought to understand if and how the endogenous eCB 2-Arachidonoylglycerol (2-AG) contributes to stress-resilience.

Published in Nature Communications, the authors first established a role for 2-AG-CB1 receptor signaling in mediating anxiety-like behavior in mice. They then tested the effects of augmenting or depleting amygdala 2-AG in two populations of mice: one empirically determined to be stress-susceptible and the other to be stress-resilient. 2-AG augmentation both increased the overall proportion of stress-resilient mice and promoted resilience in a portion of stress-susceptible mice, while depletion expectedly increased the proportion of stress-susceptible mice and converted previously stress-resilient mice into stress-susceptible mice. Using circuit-specific electrophysiological approaches the authors demonstrated a critical role for 2-AG in the amygdala for adaptation to repeated stress, and showed that phasic 2-AG-mediated suppression at ventral hippocampal-basolateral amygdala synapses is important for promoting stress-resilience.

Together, the article establishes that 2-AG-CB1 receptor signaling in the amygdala modulates stress response, resilience, and adaptation. 2-AG deficiency may contribute to the development of neuropsychiatric and affective disorders, and augmenting 2-AG could thereby potentially serve as a novel and effective strategy for treatment and prevention of these disorders.

Learn More: Bluett RJ, Báldi R, Haymer A, Gaulden AD, Hartley ND, Parrish WP, Baechle J, Marcus DJ, Mardam-Bey R, Shonesy BC, Uddin MJ, Marnett LJ, Mackie K, Colbran RJ, Winder DG, Patel S. Endocannabinoid signalling modulates susceptibility to traumatic stress exposure. Nature Communications 2017; 8:14782

Urine Trouble: Exploring the Role of Serotonin Receptor 5-HT3 in the Development of the Lower Urinary Tract

Elaine Ritter, Graduate Student

Chronic pelvic plain and bladder dysfunction effects up to 25% of women, leading to impaired quality of life and significant medical expense. Unfortunately, for the most part, current treatment options are for the most part limited to global pain and inflammation management and have a high occurrence of adverse side effects. Furthermore, the development of more targeted, restorative therapies has been hampered by a limited understanding of LUT, lower urinary tract, development and sensory innervation.

Previous reports have suggested that the serotonin receptor 5-HT3A (Htr3a) is a key mediator of bladder sensation and function. In this study, Ritter et al. (2016) further explore the role of 5-HT3A in LUT development by using a Ht3a-GFP reporter mouse line to characterize 5-HT3A expression in developing and adult bladder-projecting dorsal root ganglia (DRG) sensory neurons.

Interestingly, Ritter et al. (2016) found 5-HT3A expression begins very early in DRG development, at 12 days post coital, suggesting a possible role for 5-HT3A in LUT sensory network development. In fact, not only was 5-HT3A-GFP expression early, it was robust, with retrograde tracing of bladder innervating neurons indicating that 5-HT3A is expressed in the majority of LUT sensory afferents. Additionally, 5-HT3A-GFP was found to colocalize with known nociceptive neuronal markers- the neuropeptides CGRP and Substance P, and the capsaicin receptor TRPV1. In addition to further illuminating the development of the LUT, the authors findings highlight the potential of 5-HT3A serotonin receptor as a candidate for more targeted LUT dysfunction treatments.

Learn More: Ritter KE1, Southard-Smith EM1. Dynamic Expression of Serotonin Receptor 5-HT3A in Developing Sensory Innervation of the Lower Urinary Tract. Front Neurosci. 2017 Jan 6;10:592. doi: 10.3389/fnins.2016.00592. eCollection 2016.

RESEARCH BRIEFS

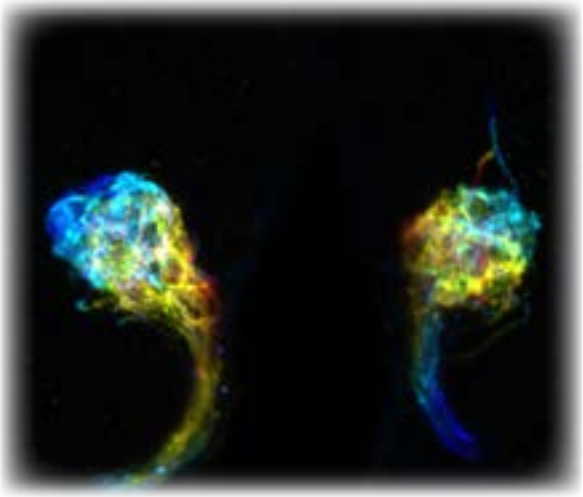
Brain Structure in early childhood can predict later functional connectivity and reasoning

Robin Schafer, Graduate Student

Previous studies have indicated an important relation between both structural and functional frontoparietal connectivity and reasoning ability. This study combined functional (functional magnetic resonance imaging) and structural (diffusion tensor imaging) neuroimaging data and measures of cognitive reasoning ability from three research sites to assess the relation of functional and structural brain connectivity to the development of reasoning ability. Additionally, this study assessed the relationship of the timing of structural and functional frontoparietal connectivity differences across development and their relation to reasoning ability. The sample included 520 participants ranging in age from 6-22 years. The authors found that functional connectivity between rostrolateral prefrontal cortex (RLPFC) and the intraparietal lobule (IPL) related to reasoning ability in adolescent and adult participants (12-22 years) but not in young children (6-11 years). Conversely, they found a relation between functional connectivity between RLPFC and the IPL in younger children but not in adolescents and adults. These findings are consistent with the developmental changes the authors observed in the structural and functional connectivity between RLPFC and IPL, whereby, greater changes in functional connectivity occur across adolescence and adulthood but greater changes in structural connectivity occur in younger children. Additionally, the authors found that stronger structural connectivity between RLPFC and IPL in young children was predictive of better reasoning ability in adolescence and adulthood. These results indicate the importance of the healthy development of frontoparietal white matter for the development of neural function and cognitive ability.

Learn More: Wendelken, Carter, Ferrer, Emilio, Ghetti, Simona, Bailey, Stephen K., Cutting, Laurie, Bunge, Silvia A. Frontoparietal Structural Connectivity in Childhood Predicts Development of Functional Connectivity and Reasoning Ability: A Large-Scale Longitudinal Investigation. Journal of Neuroscience. 2017.

On the Cover



Axon terminals of Drosophila Or42a expressing olfactory sensory neurons have been fluorescently labeled and imaged on a confocal microscope. Multiple optical sections have been superimposed and color coded based on the depth within the brain.

- Randy Golovin

Reading comprehension deficits in children: Potential applications of neurobiological language models

Katherine Aboud

Abstract

Poor reading comprehension (RC) is a major public health concern. Though RC has been implicated as a predictor of long-term educational outcomes across disciplines, up to 1/3 of children perform at below basic reading levels, meaning they are unable to extract basic information from a text (2015 National Assessment of Educational Progress). In the past several decades, much attention has been given to word decoding ability or "sounding out"; however, 10-15% of young readers struggle with RC deficits (RCD) that are independent of word decoding ability. Studies have found that readers with RCD instead exhibit semantic processing deficits (i.e. connecting words to meaning), which typically become apparent in adolescence. Despite progress in distinguishing RCD from other disorders such as dyslexia, the neural etiology of RCD is not known. Critically, neurobiological studies indicate that semantic processing is not a one-dimensional construct. Adequate RC requires appropriate (1.) retrieval of word meaning from long-term memory (semantic retrieval), and (2.) unification of the retrieved word meaning into the surrounding context (semantic integration). Functional magnetic resonance imaging (fMRI) and electroencephalography (EEG) research in adults have traced these cognitive processes to different neural systems, and found that semantic processes are marked by a key temporal component (the N400). Yet, despite considerable examination of semantic sub-processes in the adult brain, studies have not yet taken advantage of these neural models in the context of developmental reading processes. The current review discusses current neural models for semantic retrieval and integration in adults, and potential applications to developmental populations with and without RCD..

Introduction

Reading comprehension (RC) is a significant predictor of long-term performance across disciplines, including Math, Science, English and the SAT's[1]. According to the National Assessment of Educational Progress (2015), 31% of 4th graders perform at Below Basic reading levels in the U.S. This includes an estimated 10-15% of the population with reading comprehension deficits (RCD). Behavioral studies show that despite large heterogeneity across poor reader populations, children with RCD consistently struggle with semantic processing deficits (i.e. connecting words to meaning) that are independent of word decoding (i.e. "sounding out") ability. Children with RCD show deficits in measures of vocabulary knowledge[2–4], integration of semantic information within and across sentences[5,6], and executive functions that support semantic processing, such as working memory and planning/organizational ability[7,8]. These deficits often become apparent in early adolescence (10-14 year olds), during which typical reading curriculum shifts from a focus on reading fluency (i.e. "learning to read") to an emphasis on extracting meaning from texts (i.e. "reading to learn")[9]. While behavioral findings have converged on the fact that RCD is primarily a semantic processing deficit, semantic cognition is not a one-dimensional construct. Adequate semantic processing requires a reader to (1.) access word meaning (semantic retrieval), and (2.) integrate retrieved word meaning into the "message level" context of the preceding text (semantic integration)[10]. Critically, semantic retrieval and integration have not been neurobiologically characterized in typical or atypical adolescent reading. Additionally, these two processes are conflated even during simple reading behaviors, such as vocabulary measures, and as such, current behavioral models are methodologically unable to piece apart whether RCD is a retrieval deficit traced to impoverished meaning representations[2], or is a result of poor top-down integration of accessed meanings[7,11]. The development of appropriate interventions for RCD requires disentanglement of these two distinct neural processes through

neuroimaging methods. Recent work in neuroimaging has begun to elucidate the neurobiological correlates of semantic retrieval and integration during adult sentence comprehension, and consequently provide potential avenues of examination in children with RCD.

Spatiotemporal neural models of semantic retrieval and integration.

To comprehend a visual sentence, a reader has to retrieve information from long-term memory based on the phonological/orthographic word representation (e.g. semantic retrieval)[10,12]. Importantly, word meaning retrieval is not an isolated process: the local context of a word (including the other individual words in the sentence, syntax, other lexical qualities, and world knowledge) assists with the ease of retrieval through priming effects[12,13]. As an example, in the sentence "A bird spread its wings", neural access to the concept of "wings" is made easier by the preceding activation of the highly related word "bird" as well as the familiarity of the sentence content. Conversely, in the sentence "The bird landed on Mary's finger", retrieval of the word "finger" is more taxing due to a lack of a lexical prime[13]. Studies of the neural routes of semantic retrieval during sentence reading point to bilateral/left temporoparietal structures (posterior middle temporal gyrus spreading to angular gyrus; MTG and AG), which take cues from amodal semantic information in the anterior temporal lobes (ATL; Figure 1, blue arrow)[14,15].

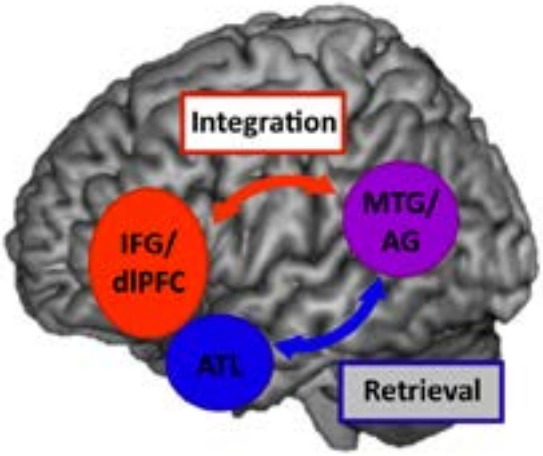


Figure 1

As readers retrieve semantic information, they also must engage in semantic integration processes. Here, "semantic integration" refers to the top-down, ongoing unification of retrieved word meanings into the evolving mental representation of the text[16]. Semantic integration involves (1.) unifying incoming meaning with the preceding text (based on information from the retrieval circuit), and (2.) subsequent constraint on upcoming semantic retrieval processes based on the evolving context (Figure 1, red arrow)[14,16–18]. A traditional and robust way to examine semantic integration is to manipulate the semantic plausibility of sentences, and consequently impede/support semantic integration (e.g. sentence "The bird spread its fingers" versus "The bird spread its wings"). Retrieval of information from long-term storage elicits a fronto-temporal semantic integration circuit[16,18,19]. Integration processes may trace to bilateral/left frontal language and executive areas (inferior frontal gyrus; (BA 45/47) and dorsolateral prefrontal cortex; IFG and dlPFC) and their potential inhibitory interactions with the retrieval circuit via the MTG/AG (Figure 1, purple region)[16,18,20]. Thus, as illustrated in Figure 1, there is significant evidence that semantic processes can be traced to iterative communication between temporal and fronto-temporal semantic retrieval and integration routes, respecitvely, in which retrieved information in temporal areas is increasingly unified and restricted by frontal areas as a text evolves.

While the spatial resolution of fMRI allows for identification of where semantic processes occur, electroencephalography (EEG) provides insight into when they occur. EEG semantic studies have primarily focused on characteristics of the N400 component, a negative waveform that occurs between 300-500 ms after stimulus onset, and which has increased amplitude for semantically incongruent stimuli [21]. This component is thought to reflect the effort of integrating a critical word into the preceding context, and more broadly, brain network activations associated with semantic memory, including semantic retrieval and integration[21,22]. Interestingly, studies have demonstrated that incongruent word pairs, sentences, and discourse all show increases in the N400 component's amplitude—an event referred to as the "N400 effect"1[3,21,23–25]. This effect is seen across age groups, though studies in children and adolescents have found that young readers have a greater N400 response across some stimuli [26].

Particularly relevant to the current study are findings that suggest that the N400 is not reflective of a singular neural source, but instead reflects a "wave" of activations across diverse areas in the semantic processing system[21]. This accounts for seemingly contradictory findings in the N400 literature, namely that the N400 appears to be associated with both automatic and controlled processes[21], and localizes to different sources depending on task[27,28], including some evidence that the N400 in word pair incongruence traces to bilateral temporal structures[27], while the N400 elicited by sentence incongruence has additionally been associated with frontal structures[28]. This array of findings has led some to suggest the N400 reflects diverse, "reverberating activity" within the full fronto-temporal semantic loop[16]. However, there is still much debate on N400 localization and more study is needed to identify spatial networks associated with the N400 across a range of tasks.

Semantic retrieval and integration in typical and atypical developing readers.

The identification of likely spatiotemporal markers of semantic retrieval and integration in adults paves the way for similar characterization in children and adolescents with and without RCD. While no studies have explicitly examined these processes in young readers, typical young readers do exhibit similar frontotemporal activations during sentence processing as adults[29–31], with some evidence for greater reliance on right hemisphere homologues[32]. In the temporal domain, N400 effect has been found to be preserved across development[26,33]. While typical semantic retrieval and integration processes in young readers may be hypothesized to parallel adult processes, less is understood about how semantic sub-processes may interact with RC ability. Traditional behavioral studies of RCD have largely examined language abilities and converged on the finding that RCD is primarily characterized by poor vocabulary ability[2]. While some have used these findings to suggest that RCD is solely an oral language deficit (suggesting RCD is traced to vulnerabilities in the retrieval circuitry; see blue arrow in Figure 1), the nature of canonical vocabulary measures conflate semantic retrieval and integration (i.e. semantic retrieval processes are inherently constrained by testing demands). Additional behavioral and neuroimaging findings demonstrate that RCD is also strongly characterized by reduced structure and function of frontal areas that others have linked to semantic integration[11,16,34]. Specifically, Bailey et al. (2016) found that compared to readers with dyslexia and typical readers, readers with RCD had reduced gray matter volume in frontal integration areas— not in the temporal semantic retrieval loop. Similarly, Aboud et al. (2016) found that during passage reading, poor readers had reduced connections between the left dlPFC and left temporoparietal areas (see Figure 2). These findings suggest that RCD may in fact be traced to vulnerabilities in semantic integration neural circuitry (see red arrow in Figure 1). However, these studies did not isolate retrieval and integration processes. ERP studies of sentence reading in adults have found that poorer readers have a reduced N400 effect during word and sentence congruency tasks[13,35,36], however, other studies have failed to find a relationship between the N400 and comprehension ability in children during word reading tasks[37]. Notably, previous ERP studies in children have been largely restricted to word reading paradigms[37,38], and the few studies on sentence reading have not examined RC ability[25,33]. Consequently, while preliminary neural evidence has begun to challenge behavioral assumptions that RCD is a semantic retrieval deficit, specific isolation of semantic retrieval and integration processes in young readers with varying reading ability is needed to build a mechanistic neurobiological model of RCD.

New methodologies in joint analysis of fMRI and EEG offer exciting opportunities to examine RCD. Specifically, joint independent component analysis[39] can be used to see which spatial networks correspond with the N400 in different semantic congruency conditions, and subsequently identify which spatiotemporal markers are disrupted in RCD. However, no studies to date have explicitly identified spatiotemporal markers of semantic retrieval and integration, or used this approach to examine RCD. Future studies should take advantage of the most recent advances in neurobiological language models and multimodal imaging techniques in order to identify brain network markers of reading comprehension deficits.

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Biomarkers of Alzheimer's Disease in the Eye and Experimental Methods of Detection via Noninvasive Ocular Imaging

Shawn Barton

Abstract

Amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs) are pathologic features of Alzheimer's disease (AD) that have been studied as biomarkers to assist in diagnosis and as a prognostic indicator for disease progression. However, many currently available techniques to visualize AD pathology are both invasive and expensive, thus limiting their utility for use in screening to identify people in early or presymptomatic stages of disease. Aβ plaques identified in both the lenses and retinas of people diagnosed with AD provide a means of visualizing AD-related pathology noninvasively. These findings have led to the development of experimental ophthalmic tests that could potentially be used as an early diagnostic tool and a way to assess therapeutic response. This review will explore the evidence supporting and arguing against the existence as well as the efficacy of targeting ocular-Aβ as a biomarker of AD. We will also discuss methods developed to visualize Aβ plaques in both the lens and retina.

Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders and is characterized by progressive memory loss, executive dysfunction, and language deficits[1]. Approximately 5.3 million Americans were living with a diagnosis of AD in 2015. Risk of developing AD increases with age, as 1 in 9 people age 65 or older (11%) are diagnosed. Prevalence increases to 1 in 3 people age 85 or older. Projections estimate that by 2025, the number of people in America age 65 or older living with AD will nearly triple to 13.8 million. Most people live an average of 4 to 8 years after being diagnosed with AD. However, some may live as long as 20 years after receiving a diagnosis. Such a long duration of illness results in significant disability and public health burden[2]. With increasing prevalence, both socioeconomic and public health impact will continue to rise unless preventative measures or therapeutics are developed.

Currently there is no cure for AD, but many studies are underway in both models of disease and in clinical trials to test novel therapeutics. The pursuit of a cure for AD has also led to investigation in identifying biomarkers specific to disease pathology. An effective biomarker could be used to assist in early clinical or even preclinical diagnosis of AD, to differentiate AD from other types of dementia that may have similar presentations, and as a prognostic biomarker to track disease progression in the presence or absence of therapy[3]. Efforts to target disease-specific pathology, including amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs) comprised of hyperphosphorylated tau aggregates, using a variety of techniques are underway. Positron emission tomography (PET), magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis for pathologic markers of disease are currently used to identify and track AD-specific biomarkers in vivo. Although there has been success in using these techniques for studying AD, current testing is often invasive, expensive, and cognitively demanding for AD patients[4]. Recent studies have identified Aβ in the eyes of AD patients, specifically in the lens[5] and the retina[6]. These findings have led to investigation in developing an ophthalmic test that is both inexpensive and noninvasive for the detection of ocular AD biomarkers. Here we review the role of NFTs and Aβ plaques in pathogenesis of AD. We will also explore studies in identification of ocular AD-pathology and efforts to develop a noninvasive ocular test.

Role of Aβ Plaques and NFTs in AD Pathogenesis

Abnormal accumulation of Aβ is a key pathologic feature of AD and the central principle of the amyloid cascade hypothesis. According to this hypothesis, aberrant processing of amyloid precursor protein (APP) results in production and poor clearance of Aβ peptide[7,8]. Aβ then aggregates in β-sheet conformations to form insoluble Aβ plaques, which cause microglial activation, cytokine release, reactive astrocytosis, inflammatory activation, and tau hyperphosphorylation leading to formation of NFTs[9-12]. Aβ deposition also results in synaptic loss and neuronal death, though the primary mechanism of toxicity is still under investigation[13]. Aβ-induced pathologic changes in particular brain regions, including the hippocampus, are thought to cause the cognitive deficits associated with AD[14]. The amyloid cascade hypothesis was further supported through studies of familial cases of AD. Mutated genes identified in studying inherited forms of AD have been shown to increase Aβ production or accumulation[15]. Also, early onset AD occurring before the age of 65 is highly prevalent in people with Down syndrome (DS) (trisomy 21), with most individuals developing AD in their 50s[16]. The APP gene is located on chromosome 21 and triplication of this chromosome in DS results in an increased gene dosage effect to accelerate Aβ plaque deposition[17].

Although Aβ plaque deposition is a key feature in most models of AD pathogenesis, total accumulation does not correlate well with clinical severity of disease[18] or brain volume loss[19]. In fact, significant Aβ plaque deposition can be found in cognitively normal elderly individuals[20]. The reason for poor correlation between Aβ plaque deposition and neuropathological changes as well as with clinical severity may be explained by the existence of soluble Aβ oligomers, which have been shown to be neurotoxic and impair synaptic activity[21, 22]. Soluble Aβ also correlates more closely with indices of disease severity[18]. These findings suggest that Aβ exists in equilibrium between soluble and insoluble states and it is the soluble form that exerts some of the neurotoxic effects that have been previously associated with Aβ plaques. Although Aβ plaque burden does not associate with disease severity, results demonstrating Aβ-mediated toxicity support the targeting of Aβ plaques as a biomarker for AD. Additionally, identification of Aβ plaques in both the lenses[5] and retinas[6] of AD patients warrant investigation in developing an ophthalmic test for identification of ocular Aβ.

Another pathologic marker identified in AD is NFT formation. NFTs are composed of hyperphosphorylated tau aggregates. Under normal conditions, tau proteins associate with microtubules and function in both microtubule assembly and stabilization, which are critical in intracellular and axonal transport. The ability of tau to associate with microtubules can be modulated via phosphorylation, where hyperphosphorylation reduces binding affinity[23]. In AD, pathologic hyperphosphorylation of tau results in intracellular aggregation to form NFTs. This is thought to disrupt axonal transport activity and lead to eventual neuronal death. Although Aβ has been shown to induce tau hyperphosphorylation[12], the way by which these two pathologic features of AD are associated remains unclear. In fact, recent evidence suggests that tau pathology may originate in the lower brainstem rather than in the transentorhinal region as previously described and may precede initial cortical plaque formation[24]. These findings suggest that both Aβ oligomer and NFT formation must occur independently for development of AD. In addition to the unclear relationship between Aβ oligomers and NFTs, tau aggregation is a pathologic feature of other diseases including frontotemporal dementia, the most common form of degenerative dementia after AD[25]. The presence of tau aggregation in other diseases limit its use as a biomarker, especially in presymptomatic stages of disease. Significant efforts have still been made to target NFTs as a biomarker using CSF testing and imaging modalities. However, tau has not been identified in the eyes of patients with AD nor has it been associated in any pathologic changes observed in the eye. Therefore, investigation in developing an ophthalmic test based on tau-mediated effects has not been pursued.

Cholinergic Deficits and Pupillary Response in AD Patients

Acetylcholine deficiency is a known feature of AD, resulting from loss of presynaptic cholinergic pyramidal neurons[4]. This observation is supported by the clinical efficacy of acetylcholinesterase inhibitors, which improve cognition and global function in AD patients[26]. An interesting early observation demonstrated that patients already diagnosed with AD exhibited marked hypersensitivity to tropicamide, a cholinergic antagonist used for pupillary dilation. Using tropicamide, variations in pupillary dilation were capable of accurately differentiating patients with AD or suspected AD from cognitively normal controls[27]. Pupillary constriction is regulated by the Edinger-Westphal nucleus, which provides preganglionic parasympathetic innervation to the eye. Treatment with tropicamide inhibits terminal signaling in this pathway and causes pupillary dilation. Exaggerated pupil dilation with cholinergic antagonist treatment in AD patients results from selective targeting of the Edinger-Westphal nucleus by AD pathology and reduced parasympathetic cholinergic signaling to the pupil[28]. Neuronal loss and AD pathology was also observed in the Edinger-Westphal nucleus of some samples from cognitively normal patients on postmortem examination. This observation may explain why some elderly subjects with normal cognition also exhibit pupillary hypersensitivity to tropicamide[29]. However, testing with tropicamide can elicit a similar hypersensitivity response in patients with vascular dementia and Parkinson's disease[30, 31]. Additionally, other studies evaluating the use of tropicamide as a diagnostic test for AD showed no significant difference in pupillary response between AD patients and healthy controls[30]. As a result of these inconsistent findings, tropicamide testing cannot be used as a diagnostic test for AD.

Evidence of Aβ in the Lens

In an effort to find a noninvasive method for detecting plaques, studies have focused on identification of Aβ in the lenses of affected individuals. However, results from these studies have produced conflicting results. Goldstein et al. were the first to identify Aβ deposition in lens specimens from individuals diagnosed with AD[5]. They identified $A\beta^{40}$ and $A\beta^{42}$ in lenses of both people with and without AD. Concentrations of both species were comparable to those seen in the cerebral cortex. On slit-lamp surveys in the same study, supranuclear cataracts were seen in all AD samples but not in the controls (Figure 1A). Supranuclear cataracts are an uncommon presentation[32] and are potentially unique to AD[33]. Histological analysis of these lenses demonstrated colocalization of supranuclear cataracts with Aβ immunoreactivity, suggesting regionally-specific Aβ aggregation in AD patients[5]. Comparable findings were seen in a study evaluating lenses from people with DS[34]. The APP gene is located on chromosome 21 and triplication of this chromosome in DS results in overexpression. Aβ accumulation occurs rapidly in those with DS[35] and results in early-onset AD in this population[36]. Those with DS also characteristically develop cerulean "blue dot" cataracts of unknown composition[37]. Moncaster et al. reported supranuclear cerulean cataracts in the lenses of DS patients that developed in an age-dependent manner[34]. Anti-Aβ immunoreactivity was observed and further localized to the cytoplasm of supranuclear fiber cells in the lens. These results suggest a common molecular origin between the increased prevalence of specific cataract phenotypes and increased prevalence of early-onset AD in people with DS and sporadic AD[5, 34]. Cataract formation is also observed in transgenic mouse models of both DS and AD[38, 39]. Interestingly, Melov et al. demonstrated that treating a transgenic mouse model of AD with the antioxidant EUK-189 resulted in significantly fewer severe cataracts compared to the untreated group[38]. This finding suggests that cataracts seen in AD form through an oxidative mechanism, which is also implicated in Aβ-related pathogenesis seen in the brain. Taken together, these results support common pathology existing in both the brain and lens in AD, particularly localized to supranuclear cataracts. Concentrations of Aβ in the lens were comparable to those seen in the brain[5] and Aβ-containing cataracts developed in an age-dependent manner in those with DS[34]. Based on these findings, plaque deposition in the lens reflects that seen in the brain and measuring Aβ concentrations in the lens could therefore serve as a biomarker for AD.

Other groups have failed to replicate the findings originally reported by Goldstein et al. in 2003. In a similarly designed study, Michael et al. examined lenses from patients diagnosed with AD, several of which had pronounced cortical lens opacities[40]. Lenses from AD donors and age-matched controls with some also containing lens opacities were stained using Congo red, thioflavin, and immunohistochemical staining but Aβ was not detected in any lens. This led to the conclusion that Aβ is not present in either AD or control cataracts[40]. The same group later published further negative results when examining the lenses of AD patients using confocal Raman microspectroscopy[41]. Using this technique they are able to measure β-sheet content in different tissues. Comparing measurements between lenses from those with AD to age-matched controls, they were unable to detect any difference in β-sheet levels within the lens even in the presence of opacities. As a control, Michael et al. measured β-sheets present in hippocampus samples from AD donors and were able to detect an increase in β-sheet levels, supporting the use of confocal Raman microspectroscopy in examining the lenses. Their findings were further supported by negative results in repeat staining analysis using Congo red, thioflavin, and immunostaining for both Aβ and tau[41]. Ho et al. were also unable to identify Aβ in the lens when stained with Congo red or immunostaining[42].

It is possible that the discrepancies between these studies were caused by differences in sample preparation. The lens is a particularly difficult structure to work with as it is more difficult to preserve than brain tissue, which was used as a positive control in the described studies. Since there was a longer postmortem interval in Michaels et al.[40], this may have reduced their ability to detect Aβ plaques. Additionally, Michaels et al. had to rely on a clinical diagnosis of AD for their lens samples as postmortem brain studies were not always possible[40, 41]. There were also differences in the sample preparation and staining protocols used, which may account for some variation. However, since there is no definitive positive control for Aβ plaque detection in the lens, it is nearly impossible to show which differences in sample preparation could account for discrepancies in the presented studies. In addition, because confocal Raman microspectroscopy was used to measure the β-sheet content of a tissue, it could be argued that Aβ in the lens has not formed β-sheet fibrils and may instead be present as soluble oligomers. This would also explain the lack of findings when staining with Congo red and thioflavin. However, absence of fibrils in the lens would not explain why Aβis not detected with specific immunostaining[41]. In summation, with such discrepancy in Aβ detection within the lens, it is controversial whether it can be considered a reliable biomarker for AD at this time.

Pursuing Aβ in the Lens as an Ocular Biomarker of AD

Although there are discrepancies in findings for whether or not Aβ is truly present in the lens of AD patients, efforts are underway to target Aβ in the lens as a biomarker for AD. One such effort, led by Lee Goldstein, is to utilize a custom-built quasi-elastic light scattering (QLS) instrument for noninvasive in vivo detection of Aβ plaques. It was previously shown that synthetic Aβ incubated with human lens protein extract potentiated aggregation and increased backscatter light intensity on QLS analysis[34]. These findings suggest that QLS analysis may be used to quantitatively assess lenses in vivo for AD pathology. Since then, QLS has been applied to noninvasively detect AD lens pathology in subjects with DS. Results have been presented at conference but are not yet published[43]. Kebege et al. have applied the SAPPHIRE system, a fluorescent ligand in combination with a laser scanning device, to detect Aβ in the human lens in vivo[44]. In this study, an ointment is prepared containing a fluorescent ligand called Compound #11, which has been shown to bind aggregated Aβ peptide[45]. The ointment was applied to a subject's eye and the lens was analyzed using a laser scanning device. Five subjects were included in the AD cohort and the cognitively normal control group. Fluorescent signal intensity from the Aβ-targeting ligand was higher in the supranuclear regions among AD patients when compared to controls[44]. These results complement the initial findings observed by Goldstein et al. that supranuclear opacifications in lenses from AD patients contained Aβ plaques[5]. Of note, one control subject determined normal by cognitive testing exhibited fluorescent measurements higher than other control subjects and may be interpreted as a false positive. This patient, though cognitively normal, had the apolipoprotein E4 variant (ApoE4). Having the

ApoE4 gene variant increases risk of developing late-onset AD[46] and therefore, the identified control subject has an increased risk of developing AD in the future. The clinical study had no serious adverse effects and results were promising that the SAPPHIRE System could in fact differentiate between those with AD and control subjects[44]. Future studies using this technique must include larger sample sizes and include longitudinal studies of subjects who are cognitively normal or with mild cognitive impairment to see if the SAPPHIRE System can be used as a screening method to predict who will later develop AD.

Evidence of Aβ in the Retina

Other research has focused on determining whether Aβ plaques form in the retina of AD patients and if detection of these could be utilized as an early diagnostic tool. The retina is a part of the central nervous system with a high density of neurons and thus may exhibit comparable Aβ accumulation as that seen in the brain. Retinal abnormalities have previously been described in AD patients including ganglion cell loss[47, 48]. Ganglion cell death was also reported in a transgenic mouse model of AD[49]. However, ganglion cell death is not a feature specific to AD[6] and thus detection would not serve as an effective biomarker for diagnosing AD. Aβ deposition has been observed in the retinas of postmortem eyes from AD patients as well as in suspected early cases using immunostaining but were not seen in age-matched controls[6]. Aβand APP immunoreactivity has also been observed in the retinas of several different transgenic mouse models of familial AD[50-53]. This deposition was shown to occur within multiple layers of the retina and in an age-dependent manner[50]. Retinal Aβ deposition in transgenic mice was shown to closely correlate with total plaque burden in the brain[6] and cause retinal degeneration as well as retinal functional impairment[50, 53]. Importantly, Aβ was detected in the retinas of transgenic mice in the presymptomatic stage and was also detected in postmortem retinal samples from people suspected to be in early stages of AD[6]. These findings suggest that an ophthalmic test designed to detect retinal Aβ could identify plaques early in AD progression and be used as an early diagnostic tool. Liu et al. also demonstrated that administration of Aβ vaccinations resulted in reduction of both brain and retinal plaque deposition in transgenic mouse models of AD[52], suggesting that noninvasive monitoring of retinal plaques could serve as a surrogate measure for total plaque burden during therapeutic trials. However, vaccination also led to an increase in Aβ in retinal vasculature[52]. Taken together, the presented studies in both human postmortem retinas and transgenic mouse models of AD provide evidence that detection of retinal Aβ could be used as an early diagnostic for AD and potentially to monitor therapeutic efficacy over the course of a clinical trial. The lack of published studies refuting Aβ in the retina as was seen within the lens suggest that detection in the retina may be a more reliable measure and pursuit of a noninvasive detection method is warranted. However, the postmortem study which identified retinal Aβ plaques in AD patients was done only for 8 cases[6]. Therefore, Aβ plaques in retinas from AD patients should be demonstrated using a larger sample size to provide more justification for pursuit of a noninvasive retinal detection test. It is also important to consider that Aβ has recently been reported in the retinas of those with age-related macular degeneration (AMD) and Aβ levels correlated with AMD progression[53]. Thus, since Aβ is present in the retina for both AMD and AD, retinal detection of Aβ in patients could potentially lead to either diagnoses and must be paired with clinical features to effectively differentiate between them, especially in early stages of disease.

In vivo Retinal imaging as an Ocular Biomarker of AD

Previous efforts have attempted to identify retinal abnormalities that may be used as a diagnostic indicator of AD. In one such study, a scanning laser ophthalmoscope (SLO), commonly used to evaluate patients with glaucoma, was used to study the optic nerve head in those with AD. It was demonstrated that patients with AD had a significant reduction in optic nerve fibers compared to control subjects[54]. Another technique used to noninvasively evaluate retinal changes in AD is optical coherence tomography (OCT), which allows for cross-sectional imaging of the retina[55]. In vivo studies using OCT has shown that peripapillary retinal nerve fiber layer thickness was significantly diminished in patients with AD[54, 56- 61] and mild cognitive

impairment[57] than cognitively normal controls. This suggests that retinal nerve fiber layer thinning may occur early in AD progression and may serve as a potential diagnostic measure. However, retinal ganglion cell death as identified using OCT has also been observed in other disorders including glaucoma[62, 63], making it less specific for early detection of AD. Measurements of retinal nerve fiber layer thickness using OCT in patients with AD did not correlate with either Alzheimer's specific CSF measurements or mini-mental status exam scores, suggesting retinal nerve fiber layer thickness may not correlate with disease progression or severity[64]. The same study by Jentsch et al. also investigated a newly developed technique of fluorescence lifetime imaging ophthalmoscopy (FLIO), which measures global changes of retinal autofluorescence. Fluorophore composition within the retina will change as a result of disease-specific accumulations and FLIO attempts to identify these changes to be used for early diagnoses and monitoring of disease progression. Jentsch et al. demonstrated that changes in FLIO parameters in AD patients compared to controls do in fact correlate with measures of AD severity[64]. However, the identified FLIO parameters may not be specific to AD. Other diseases affecting the retina must also be studied to see if measured parameters vary. Future studies using FLIO should also include patients with mild cognitive impairment who later develop AD to evaluate whether FLIO can identify changes in the retina early in disease progression. A separate study sought to identify retinal vascular biomarkers of AD by comparing retinal photographs between AD patients and healthy controls. Several retinal vascular parameters were altered in AD patients or healthy controls who demonstrated high Aβcortical plaque burden as identified using PET imaging. These results suggest that analysis of retinal vasculature could potentially be used for detection of AD or for monitoring AD progression[65]. However, none of the described techniques targeting the retina are directly measuring pathologic markers of AD.

To address this, Koronyo-Hamaoui et al. have developed a method to directly image retinal Aβ plaques noninvasively in vivo[6]. Curcumin, found in the spice turmeric, is capable of crossing the blood-brain barrier and binding cortical Aβ plaques upon intravenous injection or oral administration[66]. Additionally, curcumin's natural fluorescence allows it to be readily identified when associated with Aβ plaques. Koronyo-Hamaoui et al. administered curcumin intravenously to AD transgenic mouse models. Following administration, retinas were removed and found to have plaques labeled with curcumin as measured by fluorescence and colocalized with Aβ-specific immunostaining. Plaques were not detected in wild-type mice receiving the same curcumin injection. These findings indicate that curcumin is capable of crossing the blood-retinal barrier and associating with retinal Aβ plaques. In vivo imaging of AD transgenic mice injected with curcumin using a rodent retinal imaging microscope showed that plaques can be identified using curcumin fluorescence (Figure 1B). Curcumin fluorescence specificity was confirmed by removing the retina after retinal imaging and staining with Aβ-specific antibodies. Ex vivo results confirmed curcumin specificity using in vivo retinal imaging. Additionally, immunotherapy treatment known to reduce cortical plaque burden was also shown to reduce curcumin-visualized retinal plaques compared to non-immunized transgenic mice. Results from this study demonstrate that intravenously-administered curcumin can label Aβ plaques and be detected in vivo noninvasively[6]. This technique could potentially be used to detect Aβ plaques in patients for diagnosing AD or to monitor therapeutic efficacy in clinical trials as curcumin is known to have low toxicity even at high concentrations or with frequent dosing[67]. However, curcumin's amphiphilic properties require high levels of detergents for it to dissolve, causing the formulation to be too viscous for peripheral intravenous injection in humans[68] and thus would need to be administered via other means in clinical trials. Frost et al. reported preliminary results from a clinical trial testing the efficacy of curcumin to detect retinal Aβ plaques in AD patients. Curcumin was given orally to subjects with AD and cognitively normal controls. Retinal imaging was done to quantify curcumin fluorescence and compared to PET amyloid imaging as a measure of cortical plaque burden. Results found that curcumin retinal testing could differentiate AD patients from controls with 100% sensitivity and 80.6% specificity. The full study was expected to be completed in 2014[69] but results have not yet been published. However, curcumin has poor bioavailability due to poor absorption, rapid metabolism,

and rapid systemic elimination[70, 71]. This may reduce its efficacy in retinal Aβ detection when administered orally to humans. Recent studies have reported methods for improving curcumin's bioavailability[71] and could potentially be applied to improving noninvasive retinal plaque detection in humans

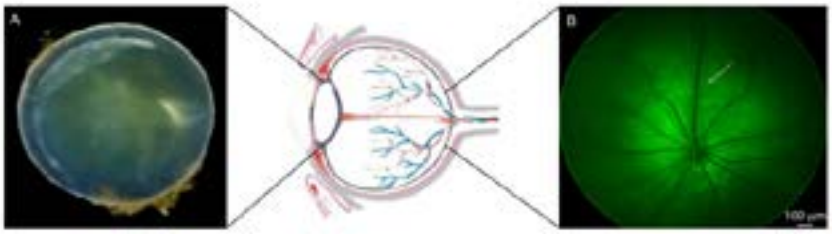

Figure 1. Ophthalmic tests targeting Aβ plaques in the lens and retina of AD patients are in development. Supranuclear cataracts seen in AD patients (A) demonstrated Aβ immunoreactivity and is a potential target for noninvasive imaging. Intravenous curcumin binds retinal Aβ plaques in the lenses of AD transgenic mice and its fluorescence was detectable noninvasively (B). Adapted from 6, 34, and 72.

Conclusion

The prevalence of AD is steadily rising and with it the need for a readily available method of detecting AD-specific pathologic features. Identification of AD pathologic deposition within the eye offers the potential for the development of an ophthalmic test that is inexpensive and noninvasive. Having such a technique will not only allow for monitoring of therapeutic efficacy during clinical trials, but also allow for identification of those with AD in early or even presymptomatic stages of disease. By identifying people in these early stages through screening, therapeutics can be initiated before significant cognitive decline has occurred and thus improve clinical outcomes as more effective treatments are developed.

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Astrocyte-Neuron Metabolic Coupling in Health and Disease

Melissa L. Cooper

Abstract

Astrocytes are a critical component of the brain, preserving neuronal health through varied conditions. They are a motile cell type, capable of remodeling to best suit the needs of surrounding cells, and a reactive line of defense against environmental variability. Astrocytes within white matter tracts, termed fibrous astrocytes, are specialized to provide structural support, energy, and protection against the stressors axons encounter. Axons are the most energy-demanding segment of a neuron, rendering them especially vulnerable to metabolic stressors. As a result, the distal axon segment is the first portion of the neuron to exhibit pathology in neurodegenerative disease. Intriguingly, morphological changes in astrocytes occur prior to these axonal changes. This is particularly apparent in the optic nerve, which contains a large, energy-demanding unmyelinated portion in the retina and through the optic nerve head. Glaucoma is an age-dependent neurodegenerative disease involving elevated intraocular pressure, which mainly stresses the edges of the optic nerve head. Despite this, early axon dysfunction is first noted distally, as in other neurodegenerative diseases. Prior to axonal changes, astrocytes redistribute their processes from the center of the nerve to the edge. This remodeling likely occurs as astrocytes reorganize to support the axons that undergo the largest degree of stress and, therefore, have the greatest metabolic need. Astrocytes are an invaluable component of neuronal metabolism because neurons and astrocytes present distinct yet complimentary metabolic profiles; astrocytes mainly utilize glycolysis, supplying neurons with lactate and pyruvate for oxidative phosphorylation. Because astrocytes can utilize energy sources dynamically to respond to neuronal needs, they are capable of compensating for most stressors. This makes neurodegenerative disease, where this compensation fails, particularly interesting. How the system changes as a result of neurodegeneration remains unclear, especially in relation to the distinct morphological changes noted in astrocytes long before axonal loss occurs.

Introduction

Astrocytes are the most abundant and diverse glial cell type in the central nervous system, distributed throughout the brain's grey and white matter, with functions as heterogeneous as their morphology[1]. They play a critical role in maintaining neuronal health both under physiological conditions and during disease. Astrocytic end-feet form and maintain the blood-brain barrier, a selective blockade that regulates the brain's extracellular environment[2]. They are responsible for recycling neurotransmitters, controlling the dynamics of cerebral blood flow, and supplying both antioxidants and energy sources to neurons[3-5].

Astrocytes are divided into two general variants defined according to the tissue in which they reside: fibrous astrocytes in white matter tracts, and protoplasmic astrocytes in grey matter[6]. Fibrous astrocytes are an integral component of white matter tracts, specialized to provide structural support, energy, and protection against the varied stressors axons encounter. Typically, more compact than protoplasmic astrocytes, fibrous astrocytes have smaller cell bodies and an elongated morphology with processes that align with the myelinated fibers they reside between. Fibrous astrocytes express elevated levels of GFAP, a type of intermediate filament, compared to protoplasmic astrocytes. Increased levels of cytoskeletal proteins allow fibrous astrocytes to provide additional support to structure myelinated fibers[7].

To monitor axons within their vicinity, fibrous astrocytes express elevated levels of the glutamate transporters GLAST[b] and GLT-1[c], allowing them to detect glutamate as it is released from the nodes of Ranvier by signaling axons[8]. Thus, astrocytes maintain a constant readout of the health and firing rate of nearby axons. They are also capable of determining the condition of nearby astrocytes through an interconnected network of gap junctions. Most astrocytes couple to adjacent astrocytes via Cx[d] 43 and, to a lesser extent, Cx 30, although this connectivity varies greatly between protoplasmic and fibrous astrocytes and further differs by brain region and cellular localization[9,10]. Due to their extensive interconnectivity and monitoring abilities, astrocytes are poised to function as a motile and reactive line of defense against environmental variability.

In the context of metabolism, fibrous astrocytes and axons are even more extensively associated. Although both astrocytes and neurons have the capacity to fully oxidize glucose, they each preferentially use different, yet complementary, metabolic pathways under physiological conditions. Astrocytes are specialized to utilize glycolysis and take advantage of the metabolic plasticity inherent in this pathway. Axons rely on large-scale energy production via mitochondria to fuel action potentials. Axons further depend upon astrocytes to provide lactate and pyruvate, the end products of glycolysis, for use in oxidative phosphorylation[11] and rely on astrocytic energy stores as a safeguard against periods of metabolic stress[12]. Considering the extensive metabolic cooperation between astrocytes and axons, it is not surprising that loss of astrocytic support hampers an axon's ability to function.

Changes in astrocytes are especially pertinent in the context of aging. Surprisingly, although neuronal changes occur, investigations of primate brains reveal a constant number of neurons from young adulthood through aging[13,14],the absolute number of myelinated axons in white matter tracts decreases dramatically[15-17]. These axonal changes throughout aging are concurrent with an increase in the volume of astrocytic processes, but not the number and proportions of glial cells[18]. Although this change may merely represent astrocytes filling in the space left by degenerating axons, there are several indications that astrocytes play a more active role in the white matter degeneration associated with age. As the brain ages, astrocyte GFAP content increases, indicative of increased reactivity[19]. Concurrently, the astrocytic secretion profile is altered and there are increased levels of general indicators of astrogliosis[20-22]. Markers of inflammation also gradually increase with age[23]. This evidence supports the hypothesis that throughout aging the proportion of white matter astrocytes in a reactive state increases.

Further alterations in astrocyte metabolic ability and morphology are related to axonal health in multiple neurodegenerative disorders. Fibrous astrocytes in multiple sclerosis show reduced ATP[e] generation and are deficient in β(2) adrenergic receptors, which are involved in stimulating glycogenolysis[24,25]. When glycolysis is inhibited and astrocytes are forced to rely upon oxidative phosphorylation in a model of alzheimer's disease, amyloid β accumulates within and around astrocytes as they become more reactive and express higher levels of GFAP[26]. Astrocyte remodeling is limited in the substantia nigra in Parkinson's disease; when astrocyte motility is stimulated via TRPV1[f] activation, endogenous production of CNTF[g] increases, preventing the degeneration of dopamine neurons and leading to behavioral recovery[27]. Together, this evidence supports a key role for astrocytes in the progression of neurodegeneration as well as underlining potential therapeutic target for the maintenance of white matter tracts.

[a]Glial Fibrillary Acidic Protein
[b]Glutamate Aspartate Transporter
[c]Glutamate Transporter 1
[d]Connexin
[e]Adenosine Triphosphate
[f]Transient Receptor Protein Vanelloid 1
[g]Ciliary Neurotrophic Factor

Glaucomatous Optic Neuropathy: A Neurodegenerative Disease

The optic nerve is a large white matter tract, containing about one million axons in humans[28]. It is the second cranial nerve, transmitting RGC impulses that code a visual scene. Terminating primarily in the lateral geniculate nucleus in humans and in the superior colliculus in rodents, the optic nerve provides the retina a route through which it can communicate with the brain. Just as in other white matter tracts, fibrous astrocytes within the nerve interact with both axons and oligodendrocytes to maintain the physiological environment and provide axons the energy needed to fire action potentials.

Unlike axons in other white matter tracts, the structure of an RGC axon renders it uniquely vulnerable to metabolic stress. As the axon passes through the retina and optic nerve head, it remains unmyelinated[28,29]. This allows light to pass through the retina unimpeded. Only after passing through the optic nerve head do axons encounter oligodendrocytes, at which point myelin is generated and nodes of Ranvier form[29]. This means that axons must fire without energy-saving saltatory conduction for a large portion of their initial length. RGC axons are also particularly small, only about 0.2-0.7 μm^2 in cross-sectional area, resulting in further inefficiency[30]. The inefficiency inherent in their structure means that astrocytic support is especially important for them to function, and renders the optic nerve a prime model for studying the interactions between astrocytes and axons in response to stress.

One unifying characteristic of age-related neurodegenerative disease is early axon dysfunction, or axonopathy, within white matter tracts[31,32]. The same is true of the optic neuropathies, the most common of which is glaucomatous optic neuropathy (glaucoma). Glaucoma is a highly prevalent age-dependent neurodegenerative disease that targets the optic nerve, the retinal ganglion cell projection to the brain, and is the leading cause of irreversible blindness worldwide[33-36]. There are two main risk factors involved in glaucomatous progression: 1) sensitivity to IOP[h], which is most likely conveyed to the unmyelinated segment of the RGC[i] axon as it passes from the retina through the optic nerve head, and 2) aging, which is a key component of most neurodegenerative diseases[37]. Elevated IOP remains the only treatable risk factor and the most predominant independent variable in animal models[38,39]. Although the mechanisms by which IOP influences the optic nerve head are complex and not completely understood, it is known that IOP does not affect the optic nerve head evenly. It appears to have its strongest effects on the edge of the nerve, where IOP has its maximum compressive effect[40].

Interestingly, although the insult first occurs at the optic nerve head, early axon dysfunction in glaucoma actually appears first in the distal optic projection[41]. Axonal dysfunction progresses distally to proximally, disassembling in a Wallerian fashion only when exposed to acute insults. Early pathogenic changes in the distal myelinated segment first include anterograde transport loss, phosphorylation of neurofilaments, and axonal enlargement[30,41]. These changes are followed much later by loss of retrograde transport, and finally degeneration of myelinated axon segments. RGC cell bodies in the retina and their presynaptic terminals within the superior colliculus persist even after degeneration of the myelinated axon segment[17]. This is especially interesting because the axon is by far the most metabolically demanding portion of a neuron and, according to the above evidence, also the most vulnerable.

Recently, evidence has emerged that early axonal dysfunction is preceded by alterations in astrocyte morphology. Prior to axonal changes, astrocytes undergo a form of remodeling that includes retraction of processes from local axon bundles and redistribution of astrocyte process density from the center of the nerve to the edge[30] (Figure 1).

[h]Intraocular Pressure
[i]Retinal Ganglion Cell

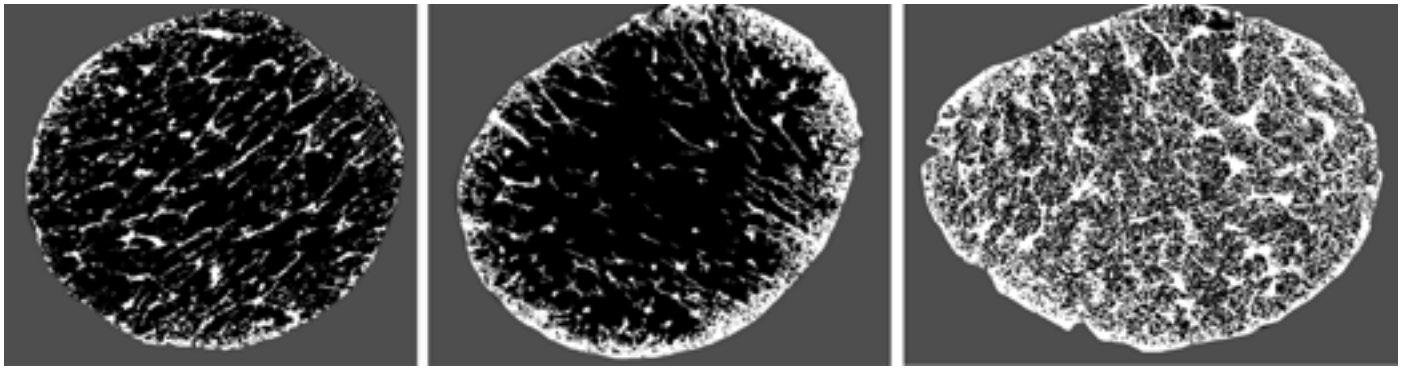

Figure 1. In a mouse model of glaucoma, cross sections of optic nerves in various stages of degeneration are represented above. As nerves progress from having a high to low axon density (left to right), glial distribution changes. In both young, healthy nerves (left) and old, unhealthy nerves (right) glial processes (white) distribute evenly across a cross section of nerve (black). However, when pathology is first noted (middle), glial processes distribute mainly to the edges of the nerve.

Astrocyte processes thicken and simplify, reducing their overall spatial coverage[42]. This early morphological remodeling appears to be reversible, and brief remodeling can occur without any discernable axonal damage[43]. After initial retraction, astrocytes sprout new processes and begin to reclaim optic nerve coverage[30,43]. This remodeling, and axonal enlargement, is concurrent with a decrease in mitochondrial density in the nerve[30]. This hints that the delicate metabolic balance between astrocytes and axons has been disturbed.

This remodeling likely occurs as astrocytes sense a stressor in their environment, redistributing their processes to best suit the nerve's needs. Astrocytes in the optic nerve head express mechanosensitive channels, allowing them to sense changes in IOP[44]. TRPV1, a well-studied mechanosensitive cation channel, is one such example that is highly expressed in astrocytes throughout the retina and brain[45,46]. When TRPV1 is agonized in vitro, the astrocyte cytoskeleton reorganizes and astrocytes migrate towards the wound; However, when antagonized, astrocyte mobilization in response to a mechanical stressor is highly attenuated[47]. Pressure exposure also causes astrocytes to release factors that abate proapoptotic signals and counteract neuronal cell death[48].

Redistribution of astrocyte processes appears to be an important protective mechanism. Even in the retina, the percent coverage of astrocytes in a sector is highly predictive of RGC health[49]. Because astrocyte process redistribution occurs prior to axonal degeneration, astrocyte reactivity may be an early response to stress that buffers against axonal loss. The exact mechanism by which astrocytes are able to protect axons is still unknown. Changes in mitochondrial density concurrent with astrocyte changes[30], along with the importance of astrocytes in metabolic support for neuronal tissue, suggest that this remodeling occurs to compensate for a metabolic need.

Metabolic Cooperation Between Astrocytes and Axons in White Matter

Astrocytes interact with nearly every neuron in the nervous system. This interaction is not merely physical; astrocytes and neurons form a single metabolic unit, compartmentalized to optimize both survival and efficiency (Figure 2). Both astrocytes and neurons have the capacity to fully oxidize glucose, but preferentially use different metabolic pathways under physiological conditions in concordance with cell-type expression patterns of key genes regulating energy metabolism[50-52]. Astrocytes largely utilize glycolysis to fuel their activities, resulting in excess lactate and pyruvate[51,53]. Neurons, consistent with the higher energy requirements needed to conduct action potentials, principally use pyruvate supplied by astrocytes and lactate within the extracellular space to fuel oxidative phosphorylation[54]. Interestingly, neurons can use lactate as an energy substrate[55], and even show a preference for lactate over glucose when both substrates are available[56,57].

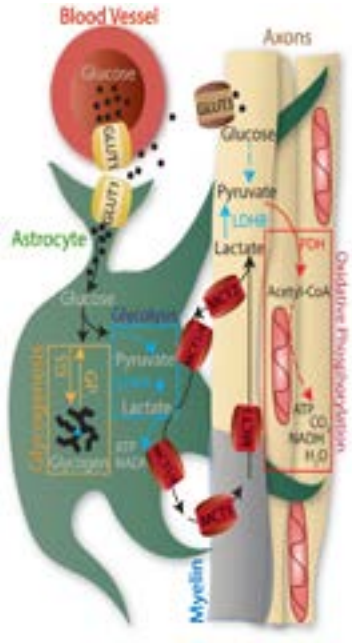

Figure 2: The astrocyte-axon metabolic unit. Both astrocytes and axons are capable of generating ATP via glycolysis, but the process is primarily utilized by astrocytes; axons take advantage of oxidative phosphorylation to generate larger volumes of ATP. Glycogenesis, however, is a process uniquely utilized by astrocytes.

This highlights the importance of nearby astrocytes in maintaining axonal metabolism. Glycolysis occurs to a limited extent in neurons, but cannot be upregulated because they lack appropriate levels of Pfkfb3[j], the enzyme that activates PFK[k], a necessary component of glycolysis. This compartmentalization and differential expression of key genes allows each cell type to optimize energy production appropriate for its needs[1]. As a result, neurons and astrocytes present distinct yet complimentary metabolic profiles. This ensures extensive metabolic cooperativity.

Astrocytes, unlike neurons, are capable of a high degree of metabolic plasticity, meaning they can adapt their energy metabolism to face various cellular challenges[58]. An example of this is the differential response of astrocytes and neurons following inhibition of mitochondrial respiration. When astrocytic mitochondrial respiration is inhibited, resulting in an energy deficit, astrocytes increase glucose metabolism through the glycolytic pathway. This limits the fall in ATP levels and prevents apoptosis. In contrast, neurons exposed to a similar challenge experience a massive ATP depletion that eventually results in apoptosis[59]. The limited metabolic plasticity of neurons is further evidenced by studies showing that driving a higher glycolytic rate[51] or glycogen synthesis[52] causes neuronal apoptosis, while astrocytes are capable of upregulating these pathways to respond to changes in their environment. When glycolysis is inhibited and astrocytes are forced to rely upon oxidative phosphorylation, amyloid β accumulates within and around astrocytes as they become more reactive and express higher levels of GFAP[26].

A key pathway metabolically plastic astrocytes utilize is glycogenesis. Glycogenesis is the process by which glucose is stored in the form of glycogen. Glycogen is the brain's largest energy reserve, and is exclusively localized in astrocytes[60,61]. Strangely, neurons do express glycogen synthase, the key enzyme in the glycogenesis pathway, but the enzyme is kept inactive through proteasomal degradation[52,61]. When glycogen metabolism is driven in cultured neurons, they undergo apoptosis – demonstrating that glycogenesis is a specific feature of astrocyte metabolism[52].

This raises an important question – why would the main energy reserve of the brain be found in astrocytes, when neurons are the more energy-demanding cell type? This is a difficult question to answer; however, this compartmentalization suggests that the interactions between astrocytes and neurons are more important than they initially appear. Neurons have the ability to increase astrocytic glycogenesis[62], which then preserves neuronal function and viability under multiple metabolically stressful conditions[61]. Astrocytes are capable of surviving while consuming little energy, so glycogen breakdown typically results in lactate and pyruvate production for neurons[63]. Under physiological conditions, increased neuronal activity induced by sensory stimulation is tightly correlated with a decrease in glycogen levels in activated white matter tracts[64,65], demonstrating the tight coupling between neuronal activity and glycogen mobilization. Additionally, astrocytic glycogen use is required to sustain retinal ganglion cell axon signaling during intense stimulation in a mouse optic nerve preparation[66,67]. Glycogen storage and breakdown, therefore, represents not only an important astrocytic protective mechanism for axons, but also a key metabolic pathway wherein the two cell types interact.

[j]6-phosphofructose-2-kinase/fructose-2,6-biphosphatase-3
[k]phosphofructokinase-1

Conclusion

Changes in the dynamic and interconnected metabolic relationship between astrocytes and neurons occur as cells age or are exposed to stressors. As neurons age, axonal transport to and from neuronal soma may not account fully for the high mitochondrial densities required to maintain distal axon terminals, especially in small axons; thus, metabolic support functions are delegated more heavily to astrocytes. As the brain ages further, metabolism becomes less efficient, starving distal axons of energy. According to this model, neurons and the cells that support them form an interconnected system that alters to compensate for aging and disease-related insults; once an insult surpasses the compensation abilities of its support system, it begins to degenerate.

This is especially important as the optic nerve degenerates in glaucoma. RGC axons are especially vulnerable to metabolic stress due to their unique structure. This highlights the importance of interactions between astrocytes and axons in the myelinated portion of the optic nerve, where degeneration is first noted. Although we do understand the metabolic interactions between astrocytes and neurons under physiological conditions, how this system changes as a result of neurodegeneration remains unclear, especially in relation to the distinct morphological changes noted in astrocytes long before axonal loss occurs.

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Cortical Multisensory Circuits: Implications for Autism Spectrum Disorder

Gabirella E. DiCarlo

Abstract

The merging of the information provided by various sensory modalities, or multisensory integration, is critical for creating healthy perceptual representations of the environment and, therefore, has significant effects on behavior. Increasing evidence suggests that alterations in multisensory processing, at the cellular and network levels, may contribute to the behavioral deficits associated with ASD. This review first introduces the principles of multisensory integration, considers the anatomical substrates underlying multisensory integration, and then discusses how changes in multisensory cortical networks may result in altered sensory processing, with an emphasis on evidence supporting the relationship between altered multisensory processing and the behavioral and social deficits associated with Autism Spectrum Disorder.

Introduction: Principles of Multisensory Processing

Stimuli in the natural world characteristically occur in more than one sensory domain. To create a coherent percept of the world, the brain must combine the information provided by each of these distinct sensory signals[1]. This process is termed multisensory integration and refers to the process by which sensory signals from separate modalities are merged into a single, unified perceptual experience[2]. Multisensory integration (MSI) is among the most critical functions of the brain as this process allows an organism to probe the environment in a variety of manners, thus enhancing both perceptual and behavioral processes[3].

Multisensory integration has been shown to occur at the level of the neuron, at the level of larger neuronal assemblages, and at the level of the behaving organism. A multisensory neuron is a neuron that responds to stimuli from more than one sensory modality (i.e. auditory and visual, visual and somatosensory, etc.). At the neuronal level, multisensory integration is defined as a statistically significant difference between the number of spikes evoked by a multimodal stimulus and the number of spikes evoked by the most effective individual stimulus alone[4]. Multisensory integration at the level of the neuron results in an output from the neuron distinct from the output elicited by the unisensory components alone. This output is often supra- or sub-additive in nature. It is important to note that this can result in either a depression or enhancement of a neuron's response. Multisensory enhancement is defined, at the behavioral level, as an increased likelihood of detecting the source of a signal, while multisensory depression refers to a decreased likelihood of detecting the source of a signal. The extent to which multisensory integration aids in the detection of an event has been shown to have a positive impact on the speed with which an organism can respond to that event. Behavioral benefits imparted by multisensory stimuli include speeded reaction times[5], enhanced target detection[5], and improved intelligibility of spoken signals[6].

At both the neuronal and behavioral levels, multisensory integration appears to follow three major principles: the temporal principle, the spatial principle, and the principle of inverse effectiveness. The temporal principle of multisensory integration states that the largest multisensory gains occur when stimuli are presented closer in temporal proximity. That is, the closer in time stimuli from distinct sensory modalities occur, the more likely those stimuli are to be perceptually bound. The spatial principle states that the closer in space two stimuli are, the greater the resulting multisensory enhancement. Stated differently, this means that that the closer in space two stimuli are, the more likely they are to be associated as originating from the same event.

Finally, the principle of inverse effectiveness states that the largest multisensory gains occur when the individual unisensory components of a stimulus are minimally effective in eliciting a behavioral or neural response alone[3]. As the salience of the component unisensory stimuli increase, the effects due to multisensory integration decrease. Multisensory depression occurs when the component unisensory events are further apart in time or space and as the strength of the unisensory stimuli increases. These principles were first established by Stein and colleagues in the superior colliculus of the cat and have since been shown to apply across many species (including rats and non-human primates) and cortical regions[1,7,8]. We will consider the anatomical regions that have been shown to contain multisensory neurons.

Anatomical Substrates of Multisensory Processing

For information from the various senses to be combined, there must exist an anatomical foundation on which this can occur. Information from the various sensory modalities is known to converge at numerous locations, both cortical and subcortical, in the central nervous system, thus providing the anatomical substrate required for multisensory integration[1]. The seminal studies in multisensory processing in the central nervous system were performed in the superior colliculus (SC) of the cat. These studies demonstrated the existence of so-called multisensory neurons (neurons that respond to stimuli from more than one sensory modality) in the deeper layers of the SC that adhered to the spatial principle of MSI, the temporal principle of MSI, and the principle of inverse effectiveness[9,10]. The deeper layers of the SC are now known to receive visual, somatosensory, and auditory inputs that arise from both ascending sensory pathways and descending cortical projections to form a two-dimensional, multisensory map of the external environment. Many of the descending inputs to the cat SC arise from unimodal neurons in the anterior ectosylvian sulcus (AES)[11]. The findings of these studies were replicated in studies of the superior colliculus in non-human primates[7].

Multisensory integration has also been shown to occur at the cortical level in cats, humans, and non-human primates. For example, the cat AES (while also projecting to multisensory neurons in the SC) has been shown to contain multisensory neurons that lie primarily at the borders between regions of unisensory neurons[12]. AES is just one of the many cortical regions that have been identified as containing multisensory neurons. Contrary to the classical view of the cortex in which each sensory modality is represented first in distinct regions, evidence indicates that multisensory neurons exist in regions of cortex typically considered to be modality-specific as well as in clusters at the borders between major cerebral lobes[8]. In rat studies, visual-auditory responsive neurons were found at the occipital/temporal border and were intermixed with modality-specific auditory and visual neurons. Similar representational patterns were found for audio-somatosensory neurons at the temporal/parietal border and visual-somatosensory neurons at the occipital/parietal border. Such multisensory neurons located in close proximity to primary sensory cortices may play a role in modulating how sensory information from each modality is processed before reaching the perceptual level. We will consider several regions known to receive multisensory inputs and their contributions to perception and behavior here.

Cortical regions shown to contain neurons receiving multisensory inputs include primary visual cortex[13], primary auditory cortex[14], and the superior temporal sulcus (STS). Work in non-human primates indicates that primary visual cortex receives input from both primary auditory cortex and from the STS[13]. Anatomical studies of primary auditory cortex have revealed afferent projections originating from primary somatosensory cortex, secondary visual cortex, the STS, and posterior parietal cortex in the Mongolian gerbil[14]. Given that the STS has been shown across many studies to receive input from both visual and auditory cortices, It is not therefore surprising that evidence suggests the STS is specialized for integrating auditory and visual speech signals[15]. Studies by Calvert demonstrated that congruent visual and auditory speech stimuli produce a greater increase in BOLD (blood-oxygen level dependent) signal in STS that would be predicted by the addition of responses to the visual or auditory stimulus alone.

That is, audiovisual congruent stimuli result in activity in STS neurons that is supra-additive with respect to the activity due to auditory or visual stimuli alone.

More recent work has shown multisensory neurons to exist both cortically and subcortically in the mouse, a convenient model for the study of the electrophysiology of the circuits related to multisensory function and the role of targeted mutations in altered sensory processing. In wild-type mice, a subpopulation of neurons responding to whisker stimulation was found to be responsive to visual stimulation in the dorsomedial striatum[16], which may indicate a role for such neurons in modulating motor output. The medial portion of V2 has been shown to receive direct input from primary visual cortex and primary auditory cortex[17], indicating a potential indirect pathway between V1 and A1 in the mouse. Such corticocortical multisensory networks may modulate the processing of sensory signals at very early stages and thus shape perceptual and behavioral outcomes.

While evidence indicates the existence of these multisensory neurons in primary sensory cortex, little work at the electrophysiological level has addressed the capacity for neurons in primary sensory cortex to integrate input from the various sensory modalities from which they receive inputs and to influence ongoing neural processing in early sensory cortices. A more complete understanding of the neurophysiology and neuroanatomy facilitating multisensory processing in these regions (such as the role of V2 in audiovisual processing and behavioral facilitation) will allow further investigation of the role that alterations in multisensory processing play in behavioral pathologies.

Autism Spectrum Disorder & Altered Sensory Processing

ASD is a highly heterogeneous neurodevelopmental disorder characterized by impairments in social communication and restricted, repetitive behaviors, interests, or activities[18]. This polygenic disorder is highly heritable, with estimates of heritability between 50-90%[19]. Symptoms begin during early development and persist into adulthood, causing impairments in social and occupational functioning that may result in difficulty achieving independence and employment in adulthood[20]. According to the most recent estimates from the CDC, the prevalence of this disorder is as high as 1 in 68 children and occurs across all socioeconomic, racial, and ethnic groups[21]. This represents a significant clinical, economic, and public health burden. Moreover, there are no medical therapies currently available for the treatment of the core symptoms of ASD.

Sensory processing challenges are a frequently reported characteristic of ASD. In fact, the most recent release of the Diagnostic and Statistical Manual of Mental Disorders includes sensory processing abnormalities as a core symptom of ASD in the diagnostic profile[18]. This feature has been reported in greater than 90% of patients of ASD, indicating a fundamental functional change in sensory processing in ASD[22]. Differences in auditory and visual processing have been reported in the literature[23–25], as have tactile hypersensitivities[26].

Due to the inherent multisensory nature of social communication and behavior, it has been hypothesized that alterations in sensory encoding and interpretation by the brain may lead to the social communication and other behavioral deficits associated with Autism Spectrum Disorder (ASD)[27]. The neurophysiological underpinnings of this disruption, however, remain unclear and remain to be more thoroughly investigated. Our current understanding of and evidence for disruptions in sensory processing at the neurophysiologic level in ASD will be considered here.

Evidence for Multisensory Processing Changes in ASD

Some literature suggests atypical early processing of auditory, visual, and tactile stimuli in individuals with ASD[28–30], as well as impairments in higher-order cortex specific for collapsing information across multiple sensory modalities in these individuals[31,32]. Functional imaging has demonstrated increased activity in ASD in brain regions associated with stimulus-driven,

sensory processing with concurrent decreased activity in regions associated with higher-order processing when compared to typically developing controls. For example, during face processing, individuals with ASD have abnormally high activity in peristriate cortex and abnormally low activity in higher association cortex known to specialize in face processing in humans (the fusiform face area)[33]. While sensory processing issues may present in various forms in ASD, typically this feature presents as sensory hyper-sensitivity, sensory hypo-sensitivity, and sensory seeking[34]. These differences in sensory processing have been correlated with symptom severity in related domains, such as social communication, in ASD[35,36]. These studies provide evidence indicating a role for altered sensory processing in producing the social and behavioral deficits associated with ASD.

Recent work suggests that multisensory function is more strongly altered in ASD compared to unisensory processing alone[37–39]. The differences in multisensory processing observed in ASD appear to be due to a reduced ability to perceptually bind information from multiple sensory modalities into a single percept and may be most impaired in conditions where the signal-to-noise ratio is low[40]. For example, evidence exists that the processing of speech (which is an inherently multisensory signal) is differentially affected in ASD when compared to processing of non-speech signals[41,42].

As described by the temporal principle of multisensory integration, perceiving multimodal stimuli (i.e. an audiovisual stimulus) as a single event depends on the temporal relationship of the cues presented, such that stimuli occurring in close temporal proximity are more likely to be perceptually bound than temporally disparate stimuli. Similarly, temporal cues can be used to segregate stimuli into discrete events. The temporal epoch during which sensory stimuli are perceptually bound has been termed the temporal binding window (TBW). Previous work has shown the TBW to be significantly larger in ASD when compared to typically developing children. That is, individuals with ASD are less likely than their typically developing peers to perceive asynchrony between an auditory and visual stimulus[38] and are less accurate at determining audiovisual temporal-order[43]. This alteration in the temporal binding of sensory stimuli further supports that changes in sensory processing and integration may underlie the core deficits associated with ASD[37,38,42].

Alterations in sensory processing in ASD are further evidenced by anatomical studies at both the cellular level and at the network level. At the cellular level, postmortem studies of individuals with ASD indicate increased columnar density in the neocortex and cerebellum, which may facilitate local (rather than global and cross-modal) processing[44,45]. It has been hypothesized that this enhanced local processing occurs at the expense of the long-range connections associated with integration and attention[46]. This is supported by diffusion tensor imaging (DTI) studies in ASD, which have demonstrated a reduced fractional anisotropy (a measure of fiber bundle organization) in ASD compared to typically developing individuals in regions associated with multisensory processing, including the superior temporal sulcus[47,48]. Functional MRI studies further support this hypothesis, as they show that the default mode network (which is active when the brain is not performing a specified task and is associated with intact connectivity), may be altered in ASD such that individuals with ASD fail to deactivate the DMN fully during speech processing (an intrinsically multisensory signal). This indicates weak coherence between disparate brain regions, further suggesting impaired functional connectivity in ASD, which may underlie altered sensory processing[49].

Conclusions

This review has presented data demonstrating the principles of multisensory integration, the possible anatomical substrates for multisensory processing, and how alterations in multisensory processing may drive the behavioral deficits associated with ASD. Much remains to be investigated in regards to the neuronal dynamics associated with multisensory processing in the cortex and the role of specific alterations in multisensory interactions in perceptual processing and behavior.

Previous work in multisensory processing in animals has been limited to larger mammals. Mice provide a useful model for the study of sensory processing and multisensory integration for a number of reasons, including the potential for targeted genetic manipulation and the ability to carry out highly precise in vivo monitoring of neuronal circuits. Strong evidence for the genetic basis of ASD has led to the development of numerous mouse models with mutations in homologous candidate genes. These mice demonstrate a behavioral phenotype consistent with the behavioral deficits associated with ASD[50]. These models make possible the study of multisensory processing and the changes in multisensory processing associated with ASD at the single-cell, network, and behavioral levels. Recent work showing behavioral facilitation under multisensory conditions in the mouse provides validation for this model for use in the exploration of the circuit mechanisms underlying multisensory behavioral facilitation[51]. Future work should aim at determining the mechanisms by which cortical multisensory neurons influence ongoing sensory processing in primary sensory cortices and whether these mechanisms can be dynamically altered. These studies will provide further evidence as to the neurophysiological basis of the behavioral deficits associated with ASD and may guide future therapies or interventions in this clinical population.

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KCC3, The Forgotten Middle Child: A Dysfunctional Role in Chloride Regulation

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Abstract

Chloride (Cl-) is the most abundant anion in the human body. As such, it works to maintain electro-neutrality in all cell types, particularly neurons. Importantly, varying intracellular [Cl-] dictates the strength of GABAergic, inhibitory synaptic networks in the nervous system. Unsurprisingly, in diseased states, like epilepsy, subsets of neurons have perturbed intracellular [Cl-]. The family of transporters responsible for maintaining proper levels of [Cl-]i include the potassium chloride cotransporters (KCCs) and the sodium potassium chloride cotransporters (NKCCs). In addition to maintaining [Cl-] i these cation chloride cotransporters (CCCs) facilitate homeostatic mechanisms that mediate cell volume. More specifically, KCC3 and KCC2 are responsible for the efflux of Cl- ions and NKCC1 is responsible for the influx of Cl- ions, creating a reciprocal regulation between the sets of cotransporters. Moreover, NKCC1 and KCC2 are integral in the neuronal development process and their basal functions are well studied, while relatively little is known about KCC3. Unsurprisingly, mutations in either KCC3 or KCC2 result in severe neurological disorders; KCC3 is linked with a rare, severe peripheral neuropathy, while KCC2 is implicated in epilepsy. Most of our understanding about KCC3 revolves around diseased murine models of human neuropathy. This review assesses our current knowledge about KCC3 and how research on NKCC1 and KCC2 can lend insight on the basal functions of KCC3.

Introduction: Cation Chloride cotransporters and their regulatory role in the nervous system

In the nervous system, Cl- defines the strength of GABAergic and glycinergic inhibitory network connections, which are necessary for patterning synaptic connections in the brain's intricate network of electrical synapses.[1] These connections are part of a dynamic and delicate balance of excitation and inhibition integral for the development of synapses, and overall brain homeostasis. Unsurprisingly, disruption in the maintenance of intracellular chloride in the adult nervous system can lead to an array of nervous system disorders. One of the primary sources of chloride regulation in the nervous system is dependent on cation chloride cotransporters (CCCs) belonging to the SLC12a gene family.[2] The SLC12a gene family (SLC12a1-9) encodes 9 different CCCs and is categorized into three different functionalities: the sodium (Na+)-potassium (K+)-coupled chloride cotransporters (NKCCs- NKCC1 and NKCC2), the K+ driven chloride cotransporters (KCCs- KCC1-4), and a Na+-Cl- cotransporter (NCC)[3]. Though these three distinct functional branches make up only SLC12a1 -7, it is unclear what the exact transporting roles are of SLC12a8-9 are.[4]

In particular, SLC12a6 (KCC3) and SLC12a5 (KCC2) are responsible for the efflux of K^+ and Cl^- in neurons. Whereas KCC2 remains active in both swelling and non-cell swelling conditions, KCC3 becomes active in only in response to cell swelling in a process known as regulatory volume decrease (RVD).[5] When cell swelling occurs, the cell will release water, K^+, Cl^-, and non-essential osmolytes in order to regain normal cell volume.[6] In contrast, NKCC1 is the primary CCC for the influx of Cl^- in neurons and responding to cell shrinkage though the complementary homeostatic mechanism, regulatory volume increase (RVI). RVI causes the cell to regain volume through the uptake of Na^+, K^+, Cl^-, and water.[6] Of all the CCCs expressed in the central and peripheral nervous systems (PNS), KCC2 and NKCC1 are primary topics of research due to their involvement in the GABA developmental shift and their implications in neonatal seizures.[7]

In comparison, relatively little is known about KCC3. Interestingly, KCC2 is expressed exclusively in the central nervous system (CNS) and no other tissue system, while KCC3 is expressed in certain regions of both the CNS and PNS and is also expressed in the kidney.[8] Specifically, KCC3 and KCC2 are co-expressed in the hippocampus and cerebellum.[9] However, KCC3 is also expressed in sensory neurons in the PNS where KCC2 remains absent.[10] The purpose of the functional redundancy with KCC2-KCC3 co-expression in certain cell types is unclear. However, the main difference between KCC2 and KCC3 is that KCC2 is active under both isotonic and hypotonic conditions, whereas KCC3 is active only in hypotonic conditions.[2]

KCC3 exists in two different isoforms: KCC3a and KCC3b. The KCC3a isoform is longer and contains several phosphorylation sites for inactivation of KCC3a, whereas KCC3b has a unique amino-terminal peptide, and does not contain the aforementioned phosphorylation sites.[11] Moreover, KCC3a is expressed in the nervous system as well as the heart and skeletal muscle, while KCC3b expression is restricted to the kidney.[12] Several studies have confirmed KCC3a as the only expressed isoform of KCC3 in the nervous system.[13,9,14] The focus of this review will remain on KCC3a, but for the remainder of this review KCC3a will be annotated as KCC3, as KCC3b is not expressed in the brain.

As one of the primary regulators of $[Cl^-]_i$ in the CNS and PNS, a disruption in KCC3 results in a rare, severe hereditary neuropathy, termed hereditary sensorimotor neuropathy with agenesis of the corpus callosum (HMSN/ACC).[15] This review will center on KCC3's current, known functional role in the nervous system, murine models that model the human disease, and implications associated with its isoform KCC2.

KCC3: Its structure, function, and expression

The structure of KCC3 is composed of 12 transmembrane spanning domains with cytoplasmic amino and carboxyl termini.[3] In addition, N-linked glycosylation sites[16] exist between transmembrane loop 5 and 6.[9] Therefore, KCC3 is considered a glycoprotein that must be transported to the plasma membrane. Glycosylation is required for KCC3 to be trafficked through the endoplasmic reticulum and golgi to make it to its final destination in the plasma membrane.[17]

Studies confirming the necessity of KCC3 glycosylation found that a single cloning mutation that changed a glutamic acid (Glu) to a Glycine (Gly) in the 289 position rendered the cotransporter functionally inactive.[17] Interestingly, this cloning mutation was in no way related to the human mutation, or disease. The Glu in the 289 position is highly conserved in CCCs, and when the corresponding Glu was mutated to Gly in KCC2 it also rendered the co-transporter non-functional in both isotonic and hypotonic conditions –conditions in which KCC2 is normally active. In contrast, this mutation only partially affected transport in NKCC1.[17] Thus, this particular site was concluded to be of greater importance to the KCCs than to NKCC1, as it appeared to be integral for glycosylation to be trafficked to the plasma membrane.

In terms of folding structure, cross-linking and FRET studies[18,19] indicate that the quaternary structure of the KCC3 may function as homodimers.[20,17] This is important as it is hypothesized that the KCC3 mutation that results in HMSN/ACC may have lost its full ability to homodimerize and thus function properly.[15] The tertiary structure of KCC3 is not well known, due to the difficulty in crystallizing the family of proteins to which it belongs. To date, the only successfully crystallized structure is the C-terminal domain of a prokaryotic CCC.[21]

KCC3 expression in the central and peripheral nervous system

KCC3 expression in the nervous system displays a wide range of expression patterns in the mouse CNS.[14] Interestingly, a microarray analysis of KCC3 from human brain tissue demonstrated that KCC3 was present in multiple regions of the CNS including the hippocampus, amygdala, and primary motor cortex, but exhibited varying spatiotemporal expression patterns.[22] The highest expression of KCC3 is observed in myelinated white matter tracts, and is found in Purkinje neurons and their axons[9]. There also appears to be KCC3 expression in mouse motor neurons.[14]

Unlike KCC2, which is absent in the PNS, KCC3 and NKCC1 are abundantly expressed.[23,24] Additionally, KCC3 expression is present in parvalbumin positive neurons. Mice with a deletion of KCC3 in parvalbumin positive neurons displayed a severe locomotor deficit and a peripheral neuropathy-like phenotype.[25] Therefore, this suggests KCC3 plays a critical role in homeostatic function in parvalbumin positive neurons.

During maturation (i.e. early post-natal days into adulthood) dorsal root ganglion (DRG) display preferential upregulation of KCC3 expression compared to other CCCs, like KCC2.[24] It is not well understood why a preferential upregulation of KCC3 occurs specifically in the DRG, but it may pertain to the fact that KCC3 is one of the primary transporters in the PNS that works to efflux Cl-, as KCC2 is not expressed in this region.

Overall, neurons, as opposed to any other nervous system cell type, appear to predominantly express KCC3.[14] This an important observation because it indicates that neuronal deficits in KCC3 may be mainly responsible for the HMSN/ACC pathology and phenotype. Ultimately, this dictates where strategies for developing potential therapeutics should focus.

Regulation of KCC3

KCC3 is considered an electroneutral protein as it moves an equal number of anions and cations across the plasma membrane, therefore creating no change in the transmembrane potential.[8] The energy it harnesses to move anions stems from the cation gradient generated by the Na-K-ATPase.[22] By its very nature, KCC3 dictates cell volume and $[Cl^-]_i$ without disrupting the normal electrical activity of a neuron[20]. It does so by sensing cell swelling and decreasing cell volume. However, there are more regulatory factors involved for KCC3 other than becoming activated in response to cell swelling. In addition to cell swelling, KCC3 is also affected by phosphorylation.[26] KCC3 contains three different phosphorylation sites: Thr^{991}, (site 1), Thr^{1048} (site 2), and Ser^{96} (site 3). All three sites must be dephosphorylated in order for KCC3 to become active. Site 1 and Site 2 are located in the carboxyl terminus,[27,28] while site 3 is located in the N-terminus.[29] As many of these sites are conserved among KCCs, the aforementioned regulation also exists in KCC2, with the exception being that KCC2 does not have a Serine active site in its N-terminus.

A set of kinases, known as the WNK (with no lysine K)-SPAK/OSR1 (SPS1-related proline/alanine-rich kinase/oxidative stress responsive kinase 1) complex, play a direct role in the phosphorylation and inactivation of KCC3. WNK activates the kinases SPAK/OSR1; the SPAK/OSR1 then complexes with mouse protein-25 (Mo25) to directly phosphorylate KCC3, inhibiting its activity **(Figure 1)**. Specifically, WNK1 activity regulates Site1 phosphorylation whereas WNK-SPAK/OSR1 with Mo25 regulate Site2.[27] Moreover, an increased extracellular K^+ concentration (hypotonic condition) results in rapid dephosphorylation of Site 1 and Site 2, thereby activating KCC3.[30] It is suggested that WNKs are able to rapidly respond to changes in the extracellular milieu because it can detect Cl^- changes in the extracellular environment.[27] Based on the crystal structure of the kinase domain of WNK1, it appears that WNK binds directly to a chloride ion.[31] Thus, WNK can act as a "chloride sensor" inhibiting the activity of KCC3 via phosphorylation.

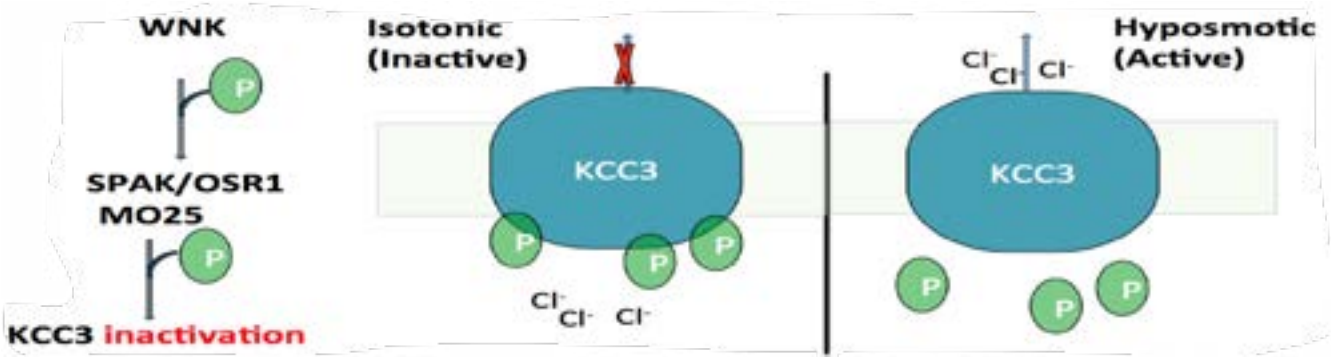

Figure 1. The WNK/SPAK/OSR1 pathway works to inactivate KCC3 via phosphorylation. WNK will phosphorylate SPAK/OSR1which then phosphorylates KCC3. KCC3 remains phosphorylated in isotonic conditions and dephosphorylated in hyposmotic conditions.

Additionally, WNK-SPAK/OSR1 complex appears to be highly specific to the KCC3 active sites. In experiments assessing point mutations of the KCC3 active sites, it was found that a dual mutation of site 1 and 2 from Thr to Ala resulted in constitutive activation (25 fold increase).[32] In spite of WNK stimulating SPAK/OSR1, the SPAK/OSR1 complex could not recognize the site to phosphorylate and inhibit KCC3 activity. This indicates that in addition to KCC3 regulating intracellular Cl^-, this phosphorylating complex adds an additional layer of regulation to indirectly affect $[Cl^-]$.

It should also be noted that reciprocal regulation of phosphorylation exists with NKCC1. As a CCC it is also regulated by the WNK-SPAK/OSR1 kinase, except it must be phosphorylated at conserved, active Thr sites in its N-terminus in order to function.[27]

The puppeteering of KCC2 and NKCC1 in the development shift and implications for KCC3

Immature neurons have a relatively high $[Cl^-]_i$ with a depolarized GABA reversal potential (EGABA-A) , while mature neurons have low levels of $[Cl^-]_i$ and hyperpolarized EGABA-A. This dramatic developmental change that occurs is often correlated with expression changes specifically in NKCC1 and KCC2.[33] KCC3 expression is low during development and decreases in the late prenatal period in humans and postnatal period in rodents[24] thus it seems unlikely that KCC3 could participate in the GABA developmental shift. This shift, in which GABA switches from depolarizing to hyperpolarizing, seems to mainly occur in central neurons where KCC2 expression is predominant.

NKCC1 exists in a type of reciprocal regulation with KCC2. In mature neurons, cell shrinkage activates NKCC1, thus NKCC1 is phosphorylated. Cell Shrinkage will also trigger phosphorylation of KCC2, thus inhibiting its activity and activating NKCC1. Throughout maturity, NKCC1 is largely responsible for the neuronal influx of Cl^- and maintaining a large $[Cl^-]_i$; comparatively, KCC2 is responsible for the neuronal efflux of Cl^- and maintaining a low $[Cl^-]_i$. This dynamic makes them both responsible for GABA's hyperpolarizing currents in the adult central nervous system. As for its role in development, NKCC1 displays a robust expression during embryonic and post-natal life and decreases once the animal reaches maturity.[22] An increased expression of KCC2 occurs simultaneously as a negative shift in EGABA is induced and as NKCC1 expression is decreasing.[7] Thus during development, this well-established "chloride-switch" changes GABA from depolarizing in immature neurons to hyperpolarizing in mature neurons.[10,24,2] Over the developmental period (neonatal to post-natal), a marked decrease $[Cl]_i$ occurs right before and after birth.[34] This is observed in the hippocampus, when NKCC1 activity is high during development, but KCC2 expression is low.[34] It should be noted that each brain region can have differing levels of NKCC1 and KCC2 robustness during the developmental period; the developmental switch occurs during the first two weeks in rodents, and late in pregnancy (prior to birth) in humans.[34] These specific changes in activity parallel with the GABA shift that occurs.[3] Overall, it is suggested that additional CCCs play a major role in this development.[24,35] Despite not directly involving KCC3, it ultimately affects how KCC3 can dictate inhibitory synaptic connections in the PNS.In sensory neurons, where KCC3 is primarily present compared to KCC2, this GABA "shift" does not seem to occur. Sensory neurons do display a marked decrease in $[Cl]_i$ but that is due to a decreased expression of NKCC1 and not necessarily an increase in KCC3 expression.[24] The change in $[Cl]_i$ appears to be under the influence of NKCC1, but not KCC3.

These important distinctions between KCC2 and KCC3 during developmental the shift give insight into the types of diseases that occur as a result of mutations in these transporters. Despite having similar roles in neurons to efflux Cl^- and maintain cell volume, perhaps it is the lack of involvement of KCC3 in this developmental process that can shed insight on why it results in a peripheral neuropathy phenotype and not necessarily disorders like seizures. For example, KCC2 plays a key role in the developmental switch, but the disruption of its regulation of intracellular chloride is associated with epilepsy.[7,36] Variants of KCC2 have has been implicated in idiopathic generalized epilepsy in which individuals that have these variants are more likely to develop seizures.[36]

Mutations in KCC2 can have an effect on the GABA shift throughout the developmental process. If KCC2 is impaired, it will likely not have proper surface expression to work in reciprocal regulation with NKCC1 during development to shift GABA from depolarizing to hyperpolarizing. Comparatively, KCC3, which has not been observed to play a major role in this GABA polarization switch, is responsible for maintaining $[Cl^-]_i$ in response to cell swelling and is not active under non-cell swelling conditions like KCC2 is. Perhaps KCC3's specific spatiotemporal expression and its absence in participation in the GABA shift make mutations in KCC3 more likely to result in peripheral neuropathy and not epilepsy.

The implications of KCC2 in neurological disorders

KCC2 is expressed in most regions of the CNS, but is absent in the PNS[37]. Originally believed not to be implicated in any human neurological disorders despite continually regulating $[Cl^-]$, KCC2 is now implicated in idiopathic general epilepsy[36]. There is also evidence that a loss of function mutation (LOF) in KCC2 is involved in the epilepsy of infancy and migrating focal seizures.[34]

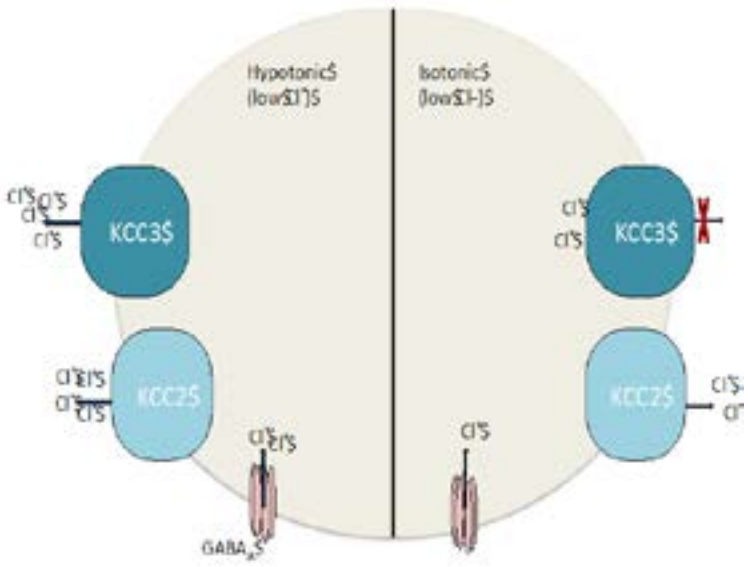

Figure 2. In hypotonic conditions KCC3 and KCC2 both work to efflux Cl^- thus promoting the influx of Cl^- through GABA. In isotonic conditions, only KCC2 will efflux Cl^-.

Unlike KCC3, KCC2 activation in both isotonic and hypotonic conditions facilitate the GABA hyperpolarizing inhibitory responses **(Figure 2)**. Interestingly, certain cell types, like pyramidal neurons, co-express KCC2 and KCC3.[3] Thus, to some degree both co-transporters act to dictate the strength of inhibitory GABAergic connections, but KCC2 predominates. However, it is unclear why two KCC isoforms would be necessary in the same cell type. What is obvious is that when either isoform is disrupted, a distinct phenotype occurs. There does not appear to be compensatory mechanisms when disruptions occur in either KCC2 or KCC3 as KCC2 null mice die soon after birth,[38] and a peripheral neuropathy phenotype occurs in KCC3 null mice.[15] However, it should be noted that patients with a KCC3 disruption mainly suffer from peripheral neuropathy, but can experience convulsions, and certain KCC3 mouse models have displayed a reduced seizure threshold.[39] It should also be noted that KCC3 has been shown to co-immunoprecipitate with KCC2,[17] hinting at some interaction between the two cotransporters.

KCC3 is responsible for one of the world's rarest peripheral neuropathies

HSMN/ACC is a severe and aggressive peripheral neuropathy that stems from a disruption in KCC3.[40] Individuals suffering from HMSN/ACC have an average life span of 33 years, are often confined to a wheel chair or bedridden, and experience hallucinatory psychosis.[15] Individuals also experience a wide ranging degree of intellectual disability, hallucinations, and frequent convulsions.[15]

Though a rare peripheral neuropathy worldwide, occurrence of HSMN/ACC occurs in 1 in approximately 2000 live births in the Quebec provinces of Charlevoix and Saguenay-Lac-St-Jean.[41] In order to develop HMSN/ACC, an individual must be homozygous for the mutation that disrupts the KCC3 C-terminus. The carrier frequency of the mutation is at an astonishingly high rate of 1 in 20 individuals in the aforementioned Canadian regions.[15] The mutation, a guanine deletion in exon 18 in the open reading frame of KCC3a, is believed to originate from a founder mutation.[42]

However, there is incidence of a non-founder haplotype mutation that exists in exon 11. There have been additional reports of a mutation in KCC3 exon 22 that also results in AC-CPN, but this individual was reported to be of Turkish Origin.[15] Ultimately, these mutations lead to a premature truncation of KCC3, rendering the cotransporter non-functional.

Mouse models of KCC3 mutations and their relation to human disease

Mouse models of disrupted KCC3 aim to mimick the human disease as closely as possible. Interestingly, none of the current mouse models exhibit agenesis of the corpus callosum (ACC). It is possible that there may be interplay with another protein, or that perhaps the murine model is not suitable for modeling disease stemming from KCC3. Overall, the following mouse models focus on the peripheral neuropathy phenotype and the cellular physiological mutations that occur.

Global Knockouts

Initial knockout models of KCC3 were designed to model the human disease by disrupting the same exon in mice that is disrupted in humans (exon 18).[15,39] In 2002, Howard, Delpire, and colleagues designed the first KCC3 knockout (KO) mouse model, Boettger et al (2003) designed the second model. Though these models were made using nearly identical knockout strategies that resulted in similar mice, there were some minor differences. These models pursued a global knockout strategy approach in which KCC3 is knocked out in every tissue system that expresses KCC3. Howard, Delpire, and collaborators were the first to publish their findings and observed a severe locomotor deficit in mice starting from 2-3 weeks of age.[15] Moreover, KCC3 KO mice displayed weakness in their rear limbs and overall a flat posture compared to their wild type counterparts. Interestingly, the heterozygous mice were indistinguishable from wild type mice. This is similar to the human disease in that an individual must be homozygous in order to display symptoms of HMSN/ACC, while a carrier (heterozygous individual) can carry the mutation but display no symptoms. In behavioral assays assessing sensorimotor gating, Howard et al. (2002) conducted a prepulse inhibition (PPI) experiment and found that KCC3 KO mice displayed a deficit in PPI. A reduced startle response is also used as a parameter to assess mouse models of schizophrenia.[43] This observation seemed to be reflective of the human disease as well since patients suffering from HMSN/ACC also suffer from Schizophrenic-like symptoms (i.e. hallucinations).[15]

Unsurprisingly, Boettger et al.'s (2003) KCC3 KO mouse model was similar in overall behavior. However, they found that their KCC3 KO mice exhibited hearing loss. It should be noted that the mice were at least 5 months, or older, when they observed this phenotype.[39] Hearing loss has not been documented in human patients, but Boettger et al.(2003) also found a reduced seizure threshold, mirroring the frequent convulsions that some individuals may experience with KCC3 mutations. Ultimately, both mouse models had the onion-bulb like nerve pathology and swollen axons similar to the human disease.[15,44]

Delpire and Byun further teased apart the underlying neuropathology in peripheral nerves using the same global KCC3 knockout model that Howard, Delpire and colleagues created. They demonstrated that, initially, sciatic nerve development appears normal compared to wild type, but axonal swelling continued throughout post-natal development (P4 and onwards). Interestingly, they observed a periaxonal fluid accumulation along the myelinated nerve, suggesting that the trapped fluid may affect the ability of Schwann cells to extrude ions and fluid.[45]

Neuronal specific knockouts

To date, there have been only two mouse models that have aimed at understanding KCC3 deletion in a neuronal specific manner. In the first specific knockout model, Shekarabi and colleagues deleted exon 18 through using a synapsin 1 promoter, which is expressed in all neuronal types. For comparison, Shekarabi et al (2012) also created a global knockout in which KCC3 was deleted in all tissues.

The global and neuronal knockout displayed similar phenotypes of neuropathology and behavior, with the exception being that the startle response was disrupted in the global knockout **(Table 1)**. It is known that neurons may play an important role in the expression and maintenance of KCC3, but since KCC3 is not necessarily expressed in all neuronal types, it is unclear if there is a set group of neurons responsible for the HMSN/ACC phenotype.

In a second neuronal specific knockout model, Delpire and colleagues designed four different mouse models, one of which included neuronal specific enolase to disrupt function in all neurons.[25] Our other models included deleting KCC3 in Schwann cells and other subsets of sensory neurons **(Table 1)**. The Delpire mouse models differ from the aforementioned mouse models in that exon 7 is disrupted. The disruption in exon 7 ultimately rendered KCC3 non-functional. Intriguingly, out of all four models, the only model that recapitulated the peripheral neuropathy phenotype was one in which the parvalbumin driven promoter was used to disrupt KCC3 in all parvalbumin positive expressing neurons. The parvalbumin-KCC3 (pv-KCC3) specific knockout resembled the initial, global knockout studies with KCC3 in that mice displayed similar severe locomotor deficits and neuropathology. Interestingly, these pv-KCC3 KO mice also exhibited a hyperactive phenotype in behavioral assays. Sub-populations of fast-spiking inhibitory interneurons are parvalbumin positive.[46] Therefore, a decrease in KCC3 activity in parvalbumin positive interneurons could lend itself to increased excitatory networks, hence a hyperactive phenotype.[25] Intriguingly, when parvalbumin is deleted in mice, they also display a tendency towards hyper-excitability and increased likelihood of epileptic seizures.[47] Therefore, this model indicates that sensory neurons and interneurons may play a role in the development of the disease. However, the hereditary neuropathy also has a motor component, thus is it has yet to be established what the effects of disrupting KCC3 specifically in motor neurons would be.

Both the global and tissue specific knockout models offer some insight on which portions of the nervous system may be responsible for the development of the disease. In spite of this information, there is still no clear understanding of how a loss of function of KCC3 contributes to development of the neuropathology and behavioral components of HMSN/ACC. Furthermore, there have not been studies to determine potential therapeutics designed specifically for KCC3. In the case of modeling the disease in mouse models, it is necessary to determine if the rescuing of the peripheral neuropathy phenotype is feasible. Moreover, it also necessary to tease apart if there are developmental components of the disease, as all previous studies have focused on the effects of a knockout from conception and observation in adulthood. Future directions for mouse models of KCC3 should investigate whether or not the HMSN phenotype is capable of rescue and should also identify precisely which populations of neurons are responsible for the progression of the disease.

Future directions for understanding the KCC3 mechanisms underlying the mutations

Despite rapid progress in the past two decades in exploring KCC3 and its implications in human disease, it is imperative that we begin to understand how KCC3 functions under basal conditions. This will include utilizing advanced genetically encoded sensors that can determine Cl^- concentration, as this could be a direct measurement of KCC3 activity. Recently, a genetically encoded sensor termed Superclomeleon has been created to accurately detect Cl^- at physiological concentrations (~5-6 mM in neurons).[48] Superclomeleon combines a chloride insensitive and a chloride sensitive chromophore to create a ratiometric measurement of chloride concentration. This application could be useful in detecting intracellular Cl^- differences between wild type KCC3 and knockout models of KCC3 with both central and sensory neurons.

Furthermore, those afflicted with HMSN/ACC have no real cure or management to the disease, but rather they have to use a multi-strategy approach to manage their symptoms. This can often include medications for hallucinations, seizures, and peripheral neuropathy; all of which, when combined, may not be optimal. We first must determine whether or not KCC3 is capable of rescue, and if there are certain developmental components that KCC3 is involved in to fully function

normally in adulthood. Even if the peripheral neuropathy induced by KCC3 mutations is incapable of rescue, this would still provide insight on the development of KCC3 in mutant conditions. Furthermore, this information would provide a different direction for the field to begin finding different strategies to alleviate varying aspects of HSMN/ACC through manipulation of KCC3.

Table 1: Overall, five successful mouse models have been made to recapitulate the peripheral neuropathy phenotype. Only one mouse model successfully deleted KCC3 in a subset of neurons in the PNS.

Lab / Reference	Disruption	Phenotype	Pathology
Delpire *Howard et al. 2002*	*Exon 3* Global KO	Locomotor deficit Crossed hind limbs	Swollen axons Onion bulb nerves
Jentsch *Boettger et al. 2003*	*Exon 3* Global KO	Locomotor deficit Crossed hind limbs Deafness	Swollen axons Onion bulb nerves
Rouleau *Shekerabi et al. 2012*	*Exon 18* -Neuron-specific synapsin-1	-Locomotor deficit -Crossed hind limbs -Deafness -Startle response disrupted	Swollen axons Onion bulb nerves
	Global KO	-Locomotor deficit -Crossed hind limbs -Deafness	Swollen axons Onion bulb nerves
Delpire *Ding & Delpire, 2014*	*Exon 7* Parvalbumin	-Locomotor deficit -Crossed hind limbs	Swollen axons Onion bulb nerves
	NaV1.8 (expressed in DRG)	No phenotype	Normal
	Desert Hedgehog (expressed in Schwann cells)	No phenotype	Normal
	Enolase-2 (mature cytosolic protein)	No phenotype	Normal

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Differential modulation of AMPAR function by auxiliary subunits

Aichurok Kamalova

Abstract

Ionotropic glutamate receptors are ligand-gated ion channels that mediate excitatory synaptic transmission in the brain. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) belong to this class of glutamate receptors, and have long been considered the "work-horses" of fast excitatory neurotransmission in the central nervous system (CNS). Significant advances over the last decade have implicated AMPARs as major determinants of synaptic strength. AMPAR auxiliary subunits are transmembrane proteins that stably interact with native AMPARs, affecting multiple aspects of AMPAR function in the brain. The following review focuses on the role of canonical auxiliary subunits in regulating early trafficking, synaptic clustering, and channel gating of AMPARs. This review also introduces a novel atypical auxiliary subunit with unique AMPAR regulatory functions.

Introduction

The mammalian brain is an intricate network comprising billions of neurons, each forming thousands of connections, called synapses, with other neurons. Synapses are critical junctions at which the electrical activity of a presynaptic neuron triggers the release of a neurotransmitter that binds to the receptors on a postsynaptic neuron and in turn may either inhibit or excite it. They are the functional units of complex brain circuits that encode all of our basic and higher-order brain functions, such as learning and memory, and dynamic changes in the strength of synaptic connections are postulated to underlie information storage that is essential for learning and memory.

Glutamate is the principal excitatory neurotransmitter in the CNS. At the postsynaptic site it binds to and activates ionotropic glutamate receptors, thereby mediating excitatory neurotransmission in the brain. Ionotropic glutamate receptors are pharmacologically subclassified into AMPA, N-methyl-D-aspartic acid (NMDA), and kainate receptors. A large body of work over the last decade has shown that the density of AMPARs at the synapse is a major determinant of synaptic strength[1–3]. AMPAR function at the synapse is defined by the composition of its channel pore-forming core subunits[4] (GluA1-4), alternative splicing[5], RNA editing[6], phosphorylation[7], and association with auxiliary subunits[8,9]. The following review will discuss the auxiliary subunit transmembrane proteins that selectively and avidly bind AMPARs and affect nearly every aspect of AMPAR life cycle from early trafficking to gating kinetics. Here, we will attempt to provide a brief overview of the last 16 years of work, specifically focusing on the role of canonical auxiliary subunits and a novel auxiliary subunit identified by our lab in modulating AMPAR synaptic function. For excellent reviews on AMPAR subunit distribution during development and synaptic plasticity, as well as alternative splicing and RNA editing of AMPARs, which are outside of the scope of this review, interested readers may refer to Henley et al. (2016)[10] and Penn and Greger (2009)[11], respectively.

AMPA Receptors

AMPARs are heterotetramers comprised of GluA1-4. Although these subunits are highly homologous, they have diverse C-terminal domains that determine interactions with different binding partners and which contain regions that undergo distinct posttranslational modifications[12]. Hence, the C-terminal tails are important for regulating various aspects of AMPAR function, including trafficking and stabilization, at the synapse.

The majority of AMPAR complexes in the mammalian CNS contain the GluA2 subunit[12], which determines the calcium permeability of the receptor. AMPARs lacking the GluA2 subunit are

calcium-permeable (CP-AMPARs) and have an inward rectifying current-voltage relationship.

AMPARs are characterized by their strikingly fast activation and desensitization kinetics underlying fast excitatory neurotransmission. Until 16 years ago, considerable incongruence existed between the biophysical properties of heterologous AMPARs[13] and those of native receptors[14], suggestive of the existence of modulatory functions of other proteins that associate with native receptors. Indeed, the discovery of transmembrane AMPAR auxiliary proteins provided strong evidence to support this prediction.

Stargazin: first bona fide AMPAR auxiliary subunit

The first bona fide AMPAR auxiliary subunit was identified through characterization of the stargazer mouse, which contained a spontaneous mutation in an inbred mouse line. The mutation causes a prominent phenotype of dyskinesia, severe ataxia, head-tossing, and severe spike-wave discharges (SWDs) that are characteristic of absence seizures in human patients[8,15]. Positional cloning mapped the causative mutation to a tetraspanin protein that shared 23% sequence homology with the γ-1 subunit of 1,4-dyhydropyridine (DHP)-sensitive calcium channels in skeletal muscle, and hence was named γ-2 (also known as stargazin)[16].

Despite the location of the mutation, stargazin surprisingly has only modest effects on the physiological function of calcium channels[16]. Instead, stargazer mice exhibit a complete loss of AMPAR-mediated synaptic currents at mossy fiber to cerebellar granule cell synapses[8,15]. Transfection of recombinant stargazin into cultured cerebellar neurons from stargazer mice rescues the mutant phenotype. Interestingly, a stargazin mutant lacking the C-terminus PDZ binding domain, a site that is important for protein-protein interactions, rescues extrasynaptic but not synaptic AMPAR-mediated current, suggesting that stargazin has two separate roles in trafficking AMPARs[8]. These studies established that stargazin is essential for synaptic delivery of AMPARs to the membrane in cerebellar granule cells, and that the distal PDZ-binding motif at the C-terminal tail of stargazin regulates synaptic clustering of AMPARs. In line with these results, subsequent experiments demonstrated that stargazin binds directly to PSD-95, a major scaffold at the excitatory postsynaptic site, as well as to other members of membrane-associated guanylate kinase (MAGUK) family[17]. The functional significance of the PSD-95-stargazin interaction will be later discussed in depth.

Identification of the TARP family

Given that stargazin is critical for the synaptic clustering of AMPARs in cerebellar granule cells, it is interesting to speculate whether stargazin plays a similar role in other brain regions. While stargazin expression is highest in the cerebellum, it is ubiquitously expressed throughout most of the brain[9,18]. However, in striking contrast to the complete functional loss of AMPARs in cerebellar granule cells in stargazer mice, AMPAR synaptic function is unaltered in forebrain neurons[8], indicating that there may be functional compensation or redundancy. In fact, subsequent studies identified stargazin as a founding member of a phylogenetically conserved tetraspanin protein family[19], subsequently named transmembrane AMPAR regulatory proteins (TARPs)[9]. Based on sequence homology and functional similarities, TARPs are subdivided into canonical type I (stargazin (γ-2), γ-3, γ-4, and γ-8), and type II (γ-5 and γ-7) subfamilies. Transfection of type I but not type II TARPs rescues AMPAR-mediated currents in cultured cerebellar granule cells from stargazer mice.

AMPAR auxiliary subunits are evolutionary conserved with TARP homologs identified in Danio rerio, C.elegans, Apis mellifera, and Drosophila melanogaster[20–22]. GLR-1, a C. elegans AMPAR homolog, requires TARP homologs STG-1 or STG2 and a structurally unrelated transmembrane auxiliary subunit SOL-1 for normal function[22]. Additionally, a stargazin ortholog, Cacng2a, was identified in developing zebrafish. Knockdown of Cacng2a in zebrafish Mauthner cells results in reduced AMPAR-mediated synaptic currents, suggesting that Cacng2a is critical for AMPAR function[21]. Therefore, TARPs appear to be evolutionary conserved gatekeepers of AMPAR function.

TARPs have four predicted transmembrane helices, intracellular N- and C-termini, and a large, functionally important extracellular loop between the first and second transmembrane helices.

Early biochemical and structural studies demonstrated that TARPs associate with AMPARs in both heterologous and native systems[8,23-33]. Single-particle electron microscopy studies showed that TARPs contribute to the transmembrane density of native system AMPARs suggestive of a stable native complex between TARPs and AMPARs in neurons[24].

The definitive stoichiometry of AMPAR-auxiliary subunit complexes remains to be determined, and would most likely require high resolution structural examination of the complex. However, indirect measures, such as kainate binding efficacy, have been used to estimate the number of auxiliary subunits associating with AMPARs at a given time[25]. Stargazin has previously been shown to increase AMPAR binding to kainate, a partial AMPAR agonist. To that end, AMPAR-TARP fusion proteins designed to achieve a fixed stoichiometry were used to titrate the number of TARPs associating with AMPARs[25]. Based on the results of these studies AMPAR complexes are postulated to functionally associate with zero, two, or four TARPs as determined by differential kainate efficacy on AMPARs for a given AMPAR-TARP complex.

TARPs regulate the early trafficking and gating of AMPARs

AMPAR auxiliary proteins regulate multiple aspects of the AMPAR lifecycle, including early biosynthetic pathways. AMPARs are assembled in the endoplasmic reticulum (ER), and are subject to quality control mechanisms prior to exiting the ER[12]. TARPs bind to AMPARs in the ER and facilitate their exit from the ER by promoting receptor maturation. As such, the ratio of immature to mature AMPARs in the stargazer and γ-3 KO mice is significantly increased[9, 26].

In addition to regulating AMPAR trafficking to the membrane and synaptic clustering, TARPs modulate the biophysical properties of AMPAR ion channel function. Single-channel characterization showed that TARPs increase AMPAR open channel probability, resulting in enhanced gating of AMPARs[27]. Multiple groups have demonstrated that co-expression of stargazin with AMPAR in heterologous expression systems slows AMPAR desensitization, speeds up recovery from desensitization, and decreases the rate of deactivation[27,28]. Interestingly, γ-4 and γ-8 slow decay kinetics to a greater extent than do stargazin or γ-3[29]. Given the differential expression pattern of TARPs across neuronal populations, the distinct modulatory effects of TARPs on channel gating may contribute to synapse-specific AMPAR regulatory mechanisms in the CNS. Domain swapping and mutational analyses revealed that the first extracellular loop is essential for modulating AMPAR gating[29].

Importantly, these modulatory properties of TARPs on gating kinetics hold true in neurons. Tomita and colleagues[27] infected hippocampal slice cultures with a dominant-negative stargazin mutant that was previously shown to abolish the TARP regulatory function on gating of AMPARs in heterologous systems. Overexpression of this construct resulted in a decrease in the amplitude and overall kinetics of miniature excitatory postsynaptic currents (mEPSCs) in hippocampal CA3 pyramidal neurons. Similar to what has been observed in heterologous systems, individual TARPs exert different modulatory effects on channel gating in neurons[29]. Taken together, studies using both heterologous systems and neurons have implicated TARPs as potential allosteric modulators of AMPAR function, specifically determining the kinetics of AMPAR-mediated currents. Overall, TARP-dependent modulation of channel gating results in increased charge transfer upon glutamate binding.

Functional interaction with PSD-95

One of the key features of excitatory synapses is the functional specialization of a postsynaptic membrane, juxtaposed to presynaptic release sites, called postsynaptic density (PSD). PSD is an electron-dense, approximately 60 nm thick morphological specialization packed with ionotropic receptors, including NMDAR, AMPAR, and GABAR, scaffolding proteins, cytoskeletal components, signaling enzymes, and other membrane proteins allowing for coupling receptor activation upon ligand binding with downstream signaling molecules[2]. PSD-95 is one member of the membrane associated guanylate

kinase family (MAGUK) located at the PSD. The PSD-95-stargazin interaction is particularly interesting, as a direct interaction between PSD-95 and AMPARs themselves has not been reported. An attractive postulation is that stargazin may serve as the middleman, targeting AMPARs to synapses via its interaction with PSD-95. However, the role of stargazin in regulating AMPAR trafficking appears to be more complex. Overexpression of stargazin selectively increases the number of extrasynaptic but not synaptic AMPARs[8], suggesting that the availability of PSD-95 at synapses is limited. Indeed, overexpression of PSD-95 in hippocampal neurons selectively increases synaptic AMPARs without altering total surface AMPAR levels[30]. Based on these findings, Schnell et al.[17] proposed that PSD-95 provides synaptic slots for AMPARs, and its interaction with stargazin clusters AMPARs from extrasynaptic to synaptic sites within the membrane. The authors further speculated that the extrasynaptic pool of stargazin-AMPAR complexes may be targeted to synapses in an activity-dependent manner during LTP.

More recently, Bats and colleagues[31] utilized single quantum dot imaging and fluorescence recovery after photobleaching (FRAP) to demonstrate that the interaction between stargazin and PSD-95 is essential for lateral diffusion of AMPARs from extrasynaptic sites to synapses in live hippocampal neurons. Furthermore, disruption of PSD-95-stargazin interaction with biomimetic divalent-competing ligands results in increased lateral diffusion of AMPARs[32]. Collectively, these data suggest stargazin plays a critical role in regulating trafficking of AMPARs to the synapse.

Role of TARPs in synaptic plasticity

Multiple lines of evidence suggest that activity-dependent phosphorylation of TARPs regulates the density of AMPARs at the PSD and influences synaptic plasticity. Biochemical studies have identified 9 conserved serine residues at the stargazin C-terminal tail that are heavily phosphorylated in neurons by calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) under basal conditions[33]. Infection of hippocampal neurons with a phosphomimetic version of stargazin (S9D) blocks NMDAR-dependent LTD, while infection with a phospho-null mutant (S9A) blocks the induction of LTP[33]. In addition, studies using single-particle trapping of AMPARs showed that NMDAR-dependent CaMKII activation and translocation to the synapse immobilizes AMPARs[34]. This phenomenon is dependent upon the phosphorylation of stargazin by CaMKII and its subsequent interaction with PSD-95 through the C-terminal PDZ-binding motif. Taken together, these studies show that phosphorylation of stargazin is important in regulating the trafficking of AMPARs in synapses.

To gain further insight into the molecular implications of TARP phosphorylation, Sumoika et al.[35] hypothesized that highly basic residues at the C-tail of stargazin interact with negatively charged membrane phospholipids. Indeed, it has since been shown that stargazin interacts with lipids in a phosphorylation-dependent manner, and that this interaction in turn inhibits stargazin-PSD-95 binding. Specifically, positively charged arginine residues at the stargazin C-tail interact with negatively charged phospholipids in the membrane and upon phosphorylation detach the C-tail from the membrane. Stargazin phospho-null (all nine serine residues mutated to alanine) knockin (KI) mice demonstrate a reduction in AMPAR-mediated mEPSCs. Conversely, stargazin phosphomimetic (all nine serine residues mutated to aspartic acid) KI mice have increased AMPAR synaptic activity as determined by mEPSC analysis.

More recent studies have further demonstrated that phosphorylation of serine residues at the C-tail of stargazin extends the effective length of the tail, allowing it to move away from the plasma membrane. The C-tail is then able to bind PSD-95, resulting in recruitment of AMPARs to the synapse[36]. In line with these findings, LTP results in decreased association of stargazin with the membrane in a CaMKII-dependent manner. Intriguingly, stargazin phosphorylation has been further implicated in experience-dependent plasticity. Louros et al.[37] demonstrate that phosphorylation of stargazin at the lateral geniculate nucleus (LGN) is differentially regulated by visual experience, and that it is essential for synaptic scaling in cultured neurons. Therefore, multiple lines of evidence suggest that posttranslational modifications of stargazin are essential for synaptic plasticity and experience-dependent homeostatic plasticity.

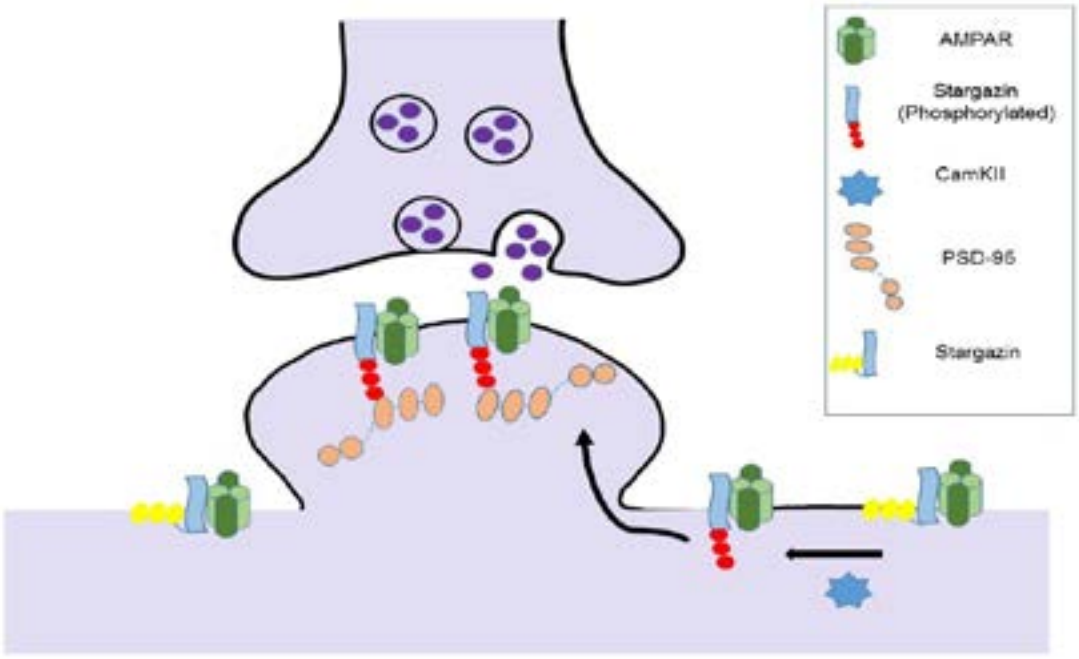

Figure 1. Stargazin-PSD-95 interaction mediates AMPAR trafficking to the synapse. Phosphorylation of the C-tail of stargazin by CaMKII disrupts the electrostatic interaction between the positively charged residues in the C-tail and the negatively charged membrane phospholipids, stargazin to bind PSD-95. This interaction results in the shuttling of AMPAR-stargazin complexes from extrasynaptic to synaptic sites.

TARP genetic models

A classic, longstanding approach to elucidate the physiological role of a protein has been to disrupt the expression of the gene encoding the protein. Interestingly, with the exception of the stargazer mouse, other TARP knockout (KO) mouse models do not present with any apparent gross behavioral defects[38]. They do, though, exhibit functional defects. For example, γ-8 KO mice display a notable reduction in AMPAR expression and distribution[39]. Synaptic AMPAR function is only modestly impaired, but there is a dramatic reduction in extrasynaptic AMPAR levels in γ-8 KO hippocampal neurons[71]. γ-8 KO mice also have decreased NMDAR-dependent LTP with no significant changes in LTD. While it is possible that the marked disruption of LTP is due to the loss of γ-8, an indirect reduction in overall AMPAR levels could be also be causing the impairment in synaptic plasticity. To address this, Sumoika and colleagues[40] developed a mutant KI mouse line that lacks the C-terminal PDZ-binding motif in γ-8. In these mice, the reduction in synaptic AMPAR levels and AMPAR-mediated EPSCs were comparable to those observed in γ-8 KO mice. However, unlike γ-8 KO mice, the mutant γ-8 KI mice have normal induction of LTP despite the loss of interaction between PSD-95 and γ-8. These results suggest that AMPAR trafficking during LTP is not dependent on the PSD-95-γ-8 interaction, which has previously been shown to be essential for AMPAR trafficking to the membrane in stargazer mice.

Interestingly, while there is a complete loss of functional AMPARs in cerebellar granule cells in stargazer mice, the function of synaptic AMPARs in the hippocampal γ-8 KO neurons is only moderately affected[39]. In situ hybridization studies have determined that the expression patterns of TARPs within the brain are widespread, but somewhat overlapping, suggestive of potential functional redundancy[18,41]. In fact, hippocampal CA1 neurons express stargazin, γ-3, γ-4, γ-7, and γ-8[18]. Therefore, it is possible that other TARP isoforms are functionally compensating for the loss of γ-8 in γ-8 KO mice, mitigating the loss of function phenotypes. This view is supported by further studies examining stargazin/γ-8 double KO mice, which present with a greater loss of synaptic AMPAR function than do γ-8 KO (50% versus 30%, respectively)[39]. It is intriguing to speculate that a quadruple stargazin/γ-3/γ-4/γ-8 KO may have a complete loss of synaptic AMPARs in hippocampal CA1 neurons. Considering the known lethality of stargazin/γ-3/γ-4, stargazin/γ-3/γ-8, and stargazin/γ-4/γ-8 triple KO models, a conditional quadruple KO will likely have to be developed[42]. Taken together, the attenuation of gene knockout phenotypes in these studies raises the possibility that TARPs functionally compensate for each other. Notably, cerebellar granule neurons are the only known population of neurons that express a single member of classical TARP family, stargazin, which could explain the strong deficits in synap

tic AMPAR function in stargazer mice. On the other hand, AMPARs in cerebellar Golgi cells, which express high levels of γ-3 in addition to stargazin, function normally in stargazer mice. γ-3 KO mice do not present with any apparent deficits in AMPAR-mediated synaptic currents in cerebellar Golgi cells, suggesting that stargazin may fully compensate for the loss of γ-3. Consistent with this premise, stargazin/γ-3 double KO mice have significant reductions in AMPAR mediated synaptic currents in addition to being sickly and highly ataxic[26,42]. Therefore, there is mounting evidence to support that TARPs function redundantly, highlighting the indispensable role for TARPs in the CNS.

An atypical AMPAR auxiliary subunit with unique modulatory functions

Following the initial discovery and characterization of stargazin and other members of the TARP family, proteomic screens and genetic data mining projects have sought to uncover novel AMPAR interactors. Our lab[43] and others[44] have recently identified an atypical AMPAR auxiliary subunit, germline-specific gene 1 like protein (GSG1L), which selectively and stably interacts with native AMPARs. GSG1L is a distant homolog of TARPs that belongs to the extended tetraspanin claudin superfamily. GSG1L is structurally similar to TARPs, with a predicted structure of four transmembrane helices, intracellular N- and C-termini, and two extracellular loops. The first extracellular loop of GSG1L is the least conserved region and is substantially longer than that of other known TARPs. Given the known role of the first extracellular loop in TARPs in modulating AMPAR gating, GSG1L was postulated to exert distinct effects on AMPAR function. Importantly, postembedding immunogold electron microscopy and immunocytochemistry has shown that GSG1L co-localizes with AMPARs in dendritic spines of cultured neurons[43,44].

Initial characterization in heterologous systems revealed that GSG1L slows desensitization and deactivation kinetics of AMPARs in a manner consistent with other TARPs[43,44]. However, unlike TARPs, GSG1L significantly slows AMPAR recovery from desensitization, and was further shown to also have unique regulatory effects on CP-AMPARs[45]. Canonical TARPs increase single-channel conductance, enhance calcium-permeability, and attenuate the intracellular polyamine block of CP-AMPARs[27,46]. Consequently, TARPs notably enhance the synaptic function of CP-AMPARs. GSG1L, on the other hand, markedly decreases the single-channel conductance and calcium permeability of CP-AMPARs[45], and enhances the polyamine block of AMPARs as was shown with recombinant AMPARs. Overexpression of GSG1L in cultured cerebellar stellate neurons, a population of neurons that doesn't normally express GSG1L, exhibited increased inward rectification of CP-AMPARs, resulting in decreased outward current from the channel. These data suggest that GSG1L is the first known auxiliary subunit with negative modulatory effects on AMPAR function. Dysregulation of CP-AMPARs has been hypothesized to contribute to cell damage after stroke and drug abuse[47]. Given the negative regulatory properties of GSG1L, it is intriguing to suppose that GSG1L may dynamically control CP-AMPAR function to mitigate excitotoxic effects of excess calcium-entry through the channel. In turn, it is possible that gain of function of GSG1L is protective against excess AMPAR activation, which would otherwise result in excitotoxicity.

A recent study by Gu et al.[48] demonstrated that GSG1L overexpression in hippocampal CA1 neurons decreases AMPAR EPSCs, highlighting their negative effect on AMPAR function. Interestingly, in striking contrast to results from heterologous systems, overexpression of GSG1L in CA1 pyramidal neurons fastened deactivation and desensitization kinetics and accelerated the recovery from desensitization. The authors speculated that the marked discrepancy with the heterologous system data is due to the presence and the regulatory effects of other molecular players in a more physiologically relevant system. In order to further interrogate the physiological role of GSG1L, Gu et al.[48] used a GSG1L KO rat model. Although there was no histological or biochemical evidence for GSG1L expression in hippocampal CA1 neurons, the results reveal an increase in AMPAR-mediated EPSCs and an enhancement of LTP in GSG1L KO CA1 neurons. Of note, in situ hybridization data showed no GSG1L expression in CA1 neurons at the developmental stage used in this study[49]. Hence, it can be argued that the observed differences in AMPAR function could be due to indirect effects of regions that communicate with CA1.

The above findings suggest that a more detailed characterization of spatial and temporal expression patterns of GSG1L is needed in order to understand the physiological role of GSG1L in regions of the brain where it is expressed. GSG1L is the only known auxiliary subunit with negative AMPAR regulatory function, and it is unknown whether there is any functional cross talk between GSG1L and TARPs under basal and/or activity-dependent conditions.

Concluding remarks

Extensive work over the last two decades has established a critical role for AMPAR auxiliary subunits in the brain. As described in this review article, AMPAR auxiliary subunits regulate multiple aspects of AMPAR function, introducing an additional layer of complexity and versatility to the physiological function of AMPARs. The loss of stargazin alone is far more detrimental than losing any or all AMPAR subunits, underscoring the importance of TARPS. Selective modulation of individual TARPs in specific brain regions may hold therapeutic potential over the non-specific modulation of AMPARs. In fact, Eli Lily and Company recently patented an AMPAR antagonist that is dependent on the presence of γ-8 to target specific brain circuits and which may be effective as an antiepileptic without the adverse side effects of known AMPAR-based treatements[50].

While we have made major leaps in understanding of the role of auxiliary subunits, many questions remain. For instance, do all AMPARs in the brain form complexes with auxiliary subunits? Can AMPAR complexes contain multiple auxiliary subunits? How is complex formation regulated when multiple auxiliary subunits are expressed in a single neuron? Given the antagonistic roles of GSG1L and canonical TARPs, there has to be some level of regulation controlling the AMPAR-auxiliary subunit complex formation when both are expressed in a single neuron. Additionally, structural studies are needed to elucidate the nature of AMPAR-auxiliary subunit complex formation.

Recent advances on the role of other auxiliary subunits are beyond the scope of this review. For a more comprehensive overview, the reader is advised to refer to excellent reviews by Yan and Tomita[51] (2010), Jackson and Nicoll[38] (2011), and Bats and Cull-Candy[52] (2013).

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Serotonin Receptors: Key Mediators of Energy Balance

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Abstract

In the last three decades, the global incidence of obesity and obesity-related diseases has more than doubled. Elucidating the mechanisms underlying the central control of feeding behavior and energy balance is essential for finding effective anti-obesity therapies. Serotonin signaling in the hypothalamus has long been known to play a role in the role in the regulation of energy homeostasis. Many decades of research have highlighted the serotonin 2C receptor as a particularly important component of the homeostatic pathways involved in energy balance. Given that the serotonin 2C receptor is a pharmacological target of several anti-obesity drugs, understanding precisely how it mediates energy balance may be crucial for the development of improved therapeutic strategies.

The maintenance of energy homeostasis requires a balance between energy intake and energy expenditure. Energy is derived from the carbohydrates, proteins, and fats in food, and it is utilized for basal metabolism, adaptive thermogenesis, and physical activity[1]. Energy status titrates a number of central and peripheral signals that work in concert to preserve energy balance[2]. Changes to either side of the energy balance equation elicit physiological responses that react to mitigate the effect of that change[1]. Dysregulation of the homeostatic mechanisms that maintain energy balance, however, results in various nutritional and metabolic diseases.

Obesity is defined as a state of increased adiposity resulting in a body mass index (BMI) more than 30kg/m^2. Although the development of obesity over time can be thought of simply as energy intake exceeding energy expenditure, the precise pathophysiology of obesity is complex as energy balance is determined by interactions between a number of genetic and environmental factors[1]. Obesity increases the risk of developing various conditions such as coronary heart disease, stroke, and type 2 diabetes, which are among the leading causes of death worldwide[3]. Between 1984 and 2014, the incidence of obesity has more than doubled[4]. This rapidly expanding public health issue has prompted efforts towards understanding the molecular framework underlying energy homeostasis and feeding behavior.

A large body of evidence demonstrates that central serotonin has an essential role in the regulation of food intake. Serotonergic action in the hypothalamus is especially important in the central control of energy balance. While several serotonin receptor subtypes are expressed within the hypothalamus, the serotonin 2C receptor has demonstrated the most significant contribution to the maintenance of energy homeostasis[5-7].

Serotonin in the control of feeding

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter that modulates a number of behaviors such as sleep, locomotion, thermoregulation, reproduction, and feeding[8]. Serotonin is synthesized within the raphe nuclei of the brainstem. These neurons project to a diverse array of targets within the central nervous system. While the caudal raphe nuclei send descending projections to the spinal cord and brainstem, the dorsal and median raphe nuclei project to the cortex, limbic areas, and the hypothalamus[9]. The first studies to reveal a role for serotonin in the control of feeding behavior suggested that serotonergic signaling promotes satiety. Central injection of serotonin or its precursor, 5-hydroxytryptophan, increases metabolic rate and suppresses feeding behavior in rodents[10-13]. This type of manipulation produces hypophagia by decreasing meal size and duration[13]. Pharmacological activation of serotonin signaling using d-fenfluramine (d-fen; discussed in detail below) also decreases food intake, but does so by reducing meal size and increasing time between meals[14]. Taken together, these results suggest that the anorectic action of serotonin works mainly to influence the termination of food consumption

(satiety) rather than meal initiation (appetite)[15]. Serotonin signaling is especially linked to a reduction in carbohydrate, but not protein intake[16]. Conversely, decreasing serotonergic tone in the brain causes hyperphagia. Specific lesions of the median raphe result in weight gain in mice[17]. Moreover, serotonin depletion following treatment with either serotonergic neurotoxin 5,7-dihydroxytryptamine, or the tryptophan hydroxylase inhibitor, p-chlorophenylalanine, induces hyperphagia and obesity in mice[18,19].

Serotonergic action in the hypothalamus

Although several brain centers are involved in the regulation of food intake and energy balance, the hypothalamus is a critical homeostatic center for many of the activities of daily life, including feeding[20]. The hypothalamus is positioned beneath the thalamus, just above the pituitary gland, and is bisected by the third ventricle[21]. The proximity of the hypothalamus to the pituitary gland, coupled with the fact that fenestrated capillaries feed the mediobasal surface, makes the hypothalamus well-suited to integrate and respond to various nutritional cues and neurotransmitters. Indeed, this anatomical arrangement permits the hypothalamus to detect circulating factors such as blood glucose and peripherally-derived hormones such as leptin, ghrelin, and insulin that would not otherwise cross the blood-brain-barrier[2,22]. In addition, the hypothalamus communicates with other brain regions involved in the control of feeding including the nucleus of the solitary tract (NTS), which receives signals from the gastrointestinal tract[22]. Through the action of many different neurotransmitters and neuropeptides, the hypothalamus conveys information about the nutritional status of the animal to regions of the brain that elicit behavioral and motor responses related to feeding.

Early studies on hypothalamic regulation of feeing behavior showed that rats with lesions to the ventromedial hypothalamus became obese[23]. Cross-circulation (parabiosis) between these obese rats and normal rats produced hyophagia and weight loss in the normal rats, suggesting that a circulating factor in the obese rats suppressed food intake[24]. Results from experiments of parabiotic pairing of the genetically obese ob/ob and db/db mouse lines with each other or with normal mice further suggested that ob/ob and db/db mice lacked a "satiety factor" and a "satiety receptor," respectively[25-27]. These hypotheses were later confirmed upon the cloning of the ob and db genes[28,29]. The ob gene encodes the adipose tissue-derived hormone leptin while the db gene encodes its cognate receptor (LEPR) [28,29]. Despite the insights gained from lesion and parabiosis studies, the limitations inherent within the crudeness of these approaches necessitated the use of more sophisticated techniques to determine the exact molecules and pathways that participate in the hypothalamic regulation of food intake and energy balance. In the last two decades, such studies have highlighted a significant contribution of serotonin signaling within a subset of neurons in the arcuate nucleus (ARC) of the hypothalamus[5,6,30].

Melanocortin feeding pathway

The melanocortin feeding pathway is a central hub responsible largely for the integration of and responses to different nutritional signals. This system comprises 1) two distinct populations of ARC neurons co-expressing either pro-opiomelanocortin (POMC) and cocaine and amphetamine related transcript (CART) or neuropeptide Y (NPY) and Agouti-related peptide (AgRP); 2) POMC-expressing neurons in the NTS; and 3) melanocortin 4 (MC4) receptor-expressing neurons, which are downstream of both POMC/CART and AgRP/NPY neurons **(Figure 1)**[22]. Post-translational enzymatic cleavage of POMC produces several peptides, one of which is alpha-melanocyte stimulating hormone (α-MSH)[31]. Interestingly, the release of α-MSH and AgRP onto MC4 receptors have antagonist effects. While agonism of MC4 receptors by α-MSH promotes satiety and decreases food intake, inverse agonism of these receptors by AgRP increases feeding. In addition, AgRP/NPY neurons inhibit POMC neurons via direct GABAergic projections. Furthermore, NPY release onto downstream neurons also induces a potent orexigenic response[2,32].

Many findings from molecular and genetic studies underscore the importance of the melanocortin pathway in the regulation of feeding and energy balance. For example, fasting increases POMC mRNA, while overfeeding decreases POMC mRNA levels[33,34]. Moreover, human or murine mutations in genes encoding POMC or the MC4R receptor result in hyperphagia and obesity[35-38]. Indeed, the most common monogenic form of

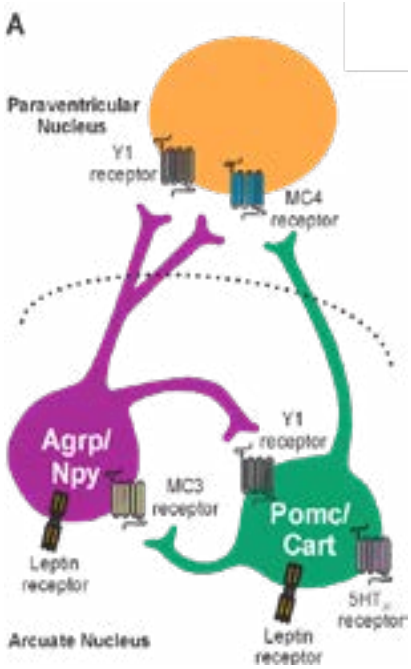

Figure 1. *Serotonin signaling in the melanocortin feeding pathway*. Activation of 5HT2C receptors stimulates the release of α-MSH from POMC neurons and promotes satiety. Release of AgRP or NPY from AgRP/NPY neurons is orexigenic. 5HT1B receptors inhibit GABA and AgRP release from NPY/AgrRP neurons. Therefore, the predominant effect of serotonin signaling in the arcuate nucleus is to suppress feeding.

early-onset obesity in humans is caused by mutations in the MC4R gene[39,40]. On the other hand, ablation of AgRP/NPY in adult mice results in starvation and AgRP mutations in humans is associated with leanness[41,42].

As coordinators of nutritional cues from the periphery and other brain regions sensitive to energy status, POMC/CART and AgRP/NPY neurons express a number of hormone, neurotransmitter, and neuropeptide receptors[2]. The vast repertoire of receptors expressed on these neurons includes, but is not limited to, leptin receptors, insulin receptors, μ opioid receptors, type 2 NPY receptors, growth hormone secretagogue receptors, and different subtypes of serotonin receptors[9,22]. Expression of these different types of receptors allows the ARC to integrate different nutritional cues, gauge energy status, and direct responses accordingly.

Serotonin receptors

Serotonin exerts its effects through interactions with fifteen different receptor subtypes which have been subdivided into seven families: $5HT_1$-$5HT_7$[43-46]. With the exception of $5HT_3$, which is a ligand-gated ion channel, all other 5-HT receptor subtypes are members of the GPCR superfamily[8]. Elucidating the role of serotonin signaling in hypothalamic feeding pathways is complicated by the expression of multiple 5HT receptor subtypes. Pharmacological, physiologic, and genetic studies have identified the $5HT_{2C}$ and $5HT_{1B}$ receptor subtypes as especially important mediators of energy balance.

$5HT_{2C}$ receptors

Of all the 5-HT receptor subtypes involved in the regulation of feeding and energy balance, the role of the $5HT_{2C}$ receptor is perhaps the most significant and best-characterized. In general, $5HT_{2C}$ receptor-mediated signaling produces anorectic effects, which is consistent with the global effects of central serotonin described above. Activation of the $5HT_{2C}$ receptor using the high affinity agonist m-chlorophenylpiperazine induced hypophagia in mice[47]. $5HT_{2C}$ receptor knockout mice display chronic hyperphagia and develop maturity-onset obesity, which are characteristics reminiscent of human forms of obesity[48,49]. While $5HT_{2C}$ receptor knockout mice were resistant to the anorectic action of d-fen, they were particularly susceptible to weight gain upon exposure to high fat diet and also to the development of insulin resistance and glucose intolerance—prominent features of type 2 diabetes[49,50].

The $5HT_{2C}$ receptor is localized almost exclusively to the CNS and also is the most abundantly expressed serotonin receptor subtype in the hypothalamus[51]. $5HT_{2C}$ receptors expressed on POMC/CART neurons of the ARC (hereafter referred to as simply POMC neurons) regulate the activity of the melanocortin pathway **(Figure 1)**. Endogenous or pharmacological activation of $5HT_{2C}$ receptors on POMC neurons causes Gq-mediated stimulation of phospholipase C (PLC) signaling[52]. The resultant increase in the intracellular second messengers diacylglycerol and inositol 1,4,5-trisphosphate ultimately leads to the release of α-MSH and in turn inhibits feeding[52]. POMC activation by 5-HT receptor agonism is blocked by disruption of $5HT_{2C}$ receptors or other perturbations of the melanocortin circuit[50]. These data suggest that $5HT_{2C}$ receptors are particularly important components of the melanocortin feeding pathway. A key experiment by Xu et al. (2008) in which 5-HT_{2C} receptors were re-expressed in an otherwise $5HT_{2C}$ receptor-null background substantiated this idea[6]. Xu et al. (2008) found that selective restoration of $5HT_{2C}$ receptors in POMC neurons is sufficient to normalize the obese and diabetic phenotypes associated

with the 5-HT_{2C} receptor knockout animals6. Furthermore, sensitivity to d-fen was also reestablished in these animals. A follow-up study showed that normal mice lacking $5HT_{2C}$ receptors only in POMC neurons developed glucoregulatory defects, hyperphagia and obesity in response to high-fat/high-sugar diet, and were resistant to the anorectic effects of d-fen[5]. Taken together, these data indicate that $5HT_{2C}$ receptors on arcuate POMC neurons are required for proper maintenance of energy homeostasis.

$5HT_{2C}$ receptors and several other types of receptors expressed on POMC neurons work in concert to fine-tune the activity of the melanocortin feeding circuit. Insulin and leptin receptor expression on POMC neurons is essential in mediating the anorixegenic effects of these hormones in the brain. A recent study found that $5HT_{2C}$ receptor-activated POMC neurons are distinct from those POMC neurons activated by leptin, and therefore suggests a segregation of the metabolic effects conferred by these signals[52]. It is unknown whether or not insulin-responsive and serotonin-responsive POMC neurons constitute the same or different populations of cells. However, based on studies of leptin receptor and $5HT_{2C}$ receptor coexpressing cells in choroid plexus, it has been suggested that insulin receptor signaling may engage the MAP kinase pathway, inhibiting the action of $5HT_{2C}$ receptor on cells expressing both kinds of receptors[53]. $5HT_{2C}$ receptors also cooperate with other subtypes of serotonin receptors within the melanocortin circuit. These relationships and their effects on feeding and energy balance are discussed below.

Adding to the complexity of $5HT_{2C}$ receptor expression in the regulation of energy balance is the fact that RNA transcripts encoding the $5HT_{2C}$ receptor are subject to post-translational processing events including alternative splicing and RNA editing. $5HT_{2C}$ transcripts under¬go up to five adenosine to ino¬sine (A-to-I) editing events that occur within a 13 base pair span of exon 5[54,55]. Editing affects the identity of three amino acids residing within the second intracellular loop of the receptor. Therefore, combinatorial editing of $5HT_{2C}$ transcripts can generate as many as 24 receptor isoforms from 32 edited mRNA species[54,55]. Experiments using heterologous expression systems reveal an inverse relationship between the extent of $5HT_{2C}$ mRNA editing and the G-coupling efficacy and constitutive activity of the receptor[56]. That is, the non-edited receptor ($5HT_{2C\text{-}INI}$) effectively couples to G-proteins and exhibits robust stimulation in the absence of bound ligand, while the fully edited receptor ($5HT_{2C\text{-}VGV}$) couples poorly to G-proteins and has very little signaling activity without bound ligand[56].

Recent evidence suggests that the differential G-coupling efficacies and constitutive activities of the $5HT_{2C}$ receptor isoforms conferred by RNA editing plays a role in $5HT_{2C}$ receptor-mediated regulation of feeding and energy balance. Mice expressing only the fully edited $5HT_{2C}$ recapitulate metabolic abnormalities, such as an early failure to thrive, post-weaning hyperphagia, and obesity, which are associated with Prader-Willi Syndrome (PWS) in humans[57]. These results suggest that normal in vivo patterns of editing are important for normal metabolic physiology. However, the "normal" $5HT_{2C}$ editing profile in POMC neurons is currently unknown. It is also unknown whether or not the $5HT_{2C}$ editing profile changes dynamically in response to fluctuations in energy status or perturbations of the melanocortin circuit.

$5HT_{1B}$ receptors

$5HT_{1B}$ receptors are expressed on AgRP neurons. Agonism of $5HT_{1B}$ receptors promotes hypophagia and satiety in rats[58]. Like $5HT_{2C}$ receptors, serotonin signaling through $5HT_{1B}$ receptors mediates anorexigenic effects in a manner dependent on the MC4 receptors[59]. Interestingly, although genetic ablation of $5HT_{2C}$ receptors causes obesity in mice, $5HT_{1B}$-null mice do not gain weight to the same degree[60]. However, disruption of $5HT_{1B}$ receptor through genetic or pharmacological means decreases sensitivity to d-fen[61]. Taken together, these data suggest cooperation between $5HT_{1B}$ and $5HT_{2C}$ receptors. Indeed, Heisler and colleagues showed that serotonin signaling through $5HT_{1B}$ receptors promotes satiety through suppression of AgRP release as well as disinhibition of POMC neurons[59]. Therefore, by inhibiting AgRP neurons and stimulating POMC neurons, serotonin works via two parallel mechanisms to suppress feeding behavior[59].

Other 5-HT receptor subtypes

Paradoxically, some studies have suggested that signaling through $5HT_{1A}$ and $5HT_{2B}$ subtypes of serotonin receptors expressed on POMC neurons mediates orexigenic effects. Agonism of $5HT_{1A}$ receptors increases food intake while antagonism of $5HT_{1A}$ receptors reduces food intake. Conditional knockout of the $5HT_{1A}$ receptor selectively in POMC-expressing cells induces early hypophagia followed by leanness at 6 months of age in mice[62]. Mice lacking $5HT_{2B}$ receptors specifically in POMC-expressing cells are also hypophagic and lean[51].

Centrally-Acting Anti-Obesity Drugs

Given the substantial health and economic burdens imposed by the obesity epidemic, development of effective therapies to combat obesity is essential. Unfortunately, prescribed diet and exercise regimens and therapies such as behavioral and nutritional counseling have modest and temporary effects at best[63]. Although these strategies will undoubtedly be useful in the long-term prevention of obesity, there is an immediate need for treatments effective in those who are already obese. Currently, bariatric surgery is the most effective treatment for obese individuals, but it is expensive, invasive, and usually intended only for morbidly obese patients[63]. For these reasons, the development of anti-obesity drugs is an attractive therapeutic strategy. Because overwhelming evidence has implicated the $5HT_{2C}$ receptor as a critical regulator of energy homeostasis, it has long been targeted for the treatment of obesity. The following sections review two $5HT_{2C}$ receptor-targeting drugs: Fenfluramine and Lorcaserin.

Fenfluramine

During the 1960s, amphetamine-like drugs were noted for their appetite-suppressing effects. By the 1990s, one of these drugs in particular, fenfluramine, became widely prescribed as an anti-obesity treatment. Consistent with the view that serotonin promotes satiety, fenfluramine works to activate serotonin signaling on POMC neurons[7]. Fenfluramine blocks the vesicular transporter and increases intracellular serotonin levels[64]. This increase in intracellular serotonin reverses the action of the serotonin transporter, resulting in serotonin efflux[65,66]. Thus, fenfluramine stimulates the release and inhibits the reuptake of serotonin. Importantly, the metabolite of fenfluramine, norfenfluramine, is a potent activator of the $5HT_{2C}$ receptor[67]. Administration of fenfluramine with selective $5HT_{2c}$ receptor antagonists blocks the anorectic effects of fenfluramine, suggesting that the main mechanism of action of fenfluramine (and norfenfluramine) is $5HT_{2C}$ receptor agonism[68,69]. Despite the clinical effectiveness of fenfluramine, it was withdrawn from the market in 1997 due to off-target stimulation of valvular $5HT_{2B}$ receptors which caused cardiovascular complications in a subset of patients[70].

Lorcaserin

The withdrawal of fenfluramine from the market in 1997 warranted the development of more selective $5HT_{2C}$ receptor agonists for the treatment of obesity. In 2012, the United States Food and Drug Administration approved the use of lorcaserin (Belviq), an anti-obesity drug with ~15 and ~100 fold greater affinity for the $5HT_{2C}$ receptor than the $5HT_{2A}$ and $5HT_{2B}$ receptors, respectively. The recommended dose (10 mg, twice daily) carries low risk of off-target effects, including valvulopathies and pulmonary hypertension. However, lorcaserin acts at $5HT_{2A}$ and $5HT_{2B}$ receptors when administered in supratherapeutic doses[71,72]. Moreover, high doses of lorcaserin were associated with tumor formation in rats[63]. These animal studies informed the initial FDA decision to reject lorcaserin in 2010. The FDA stated that the efficacy of the drug was "marginal" compared to the risk profile[63,73]. Even after receiving FDA approval, the long-term efficacy of lorcaserin is still in question. Phase 3 clinical trials found that patients receiving the therapeutic dose of lorcaserin lost an average of 5.8% of their body weight, whereas patients receiving placebo lost an average of 2.9% of their body weight[74,75]. A meta-analysis of five randomized controlled trials found that treatment with lorcaserin for 12 weeks demonstrated an average weight loss of 3 kg in a patient whose starting weight was 100 kg[76]. This translates to a reduction in BMI by 1.16 kg/m2 from a basal mean BMI of 36.1 kg/m2. Although a 5-10% reduction in body weight reduces the risk for chronic diseases related to obesity, many of the patients who do not lose more weight are still considered

overweight or obese[77]. Furthermore, it is currently unknown whether or not the effects of lorcaserin can be sustained beyond two years. Thus, despite increased safety and selectivity of lorcaserin over other $5HT_{2C}$ receptor agonists, the dose at which it is prescribed may not be sufficient to produce large, long-lasting reduction in body weight.

Conclusion

In conclusion, the $5HT_{2C}$ receptor is a key mediator of energy balance. Within the arcuate nucleus, it cooperates with a number of other receptors to regulate energy balance. Although the $5HT_{2C}$ receptor is a target of the FDA-approved drug lorcaserin, the limited therapeutic benefit of this drug has suggested that a deeper understanding of how the $5HT_{2C}$ receptor mediates energy balance is necessary to create better therapeutic strategies.

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Prefrontal cortical endocannabinoid signaling as a endogenous buffer against stress induced affective disturbances

David Marcus

Abstract

The role of the medial prefrontal cortex (mPFC) in top-down regulation of emotional behavior has been established for well over 100 years. The mPFC, which is the most highly conserved sub-region of the PFC in mammals, is a major center of information integration, receiving not only higher order sensory information but also interoceptive data conveying valence and emotional state. In this way, the mPFC often plays an important role in orchestrating the output of more 'primitive' limbic regions that are involved in processing mood, emotion, and stress. An increasing body of evidence suggests that the endocannabinoid system (ECS) acts as a central modulator of the stress response in the mPFC. To that end, targeting the ECS may be an effective strategy for the treatment of stress. The following review will detail the role of the ECS in the mPFC, and propose a model for the interaction between the ECS and stress response.

Medial prefrontal cortex regulation of the Hypothalamic-Pituitary-Adrenal Axis: a role for endocannabinoids

Although most studies have examined the mPFC as a singular functional unit, more nuanced examination of this region has revealed functionally distinct subdivisions that receive unique projections. The most dorsal aspect of the mPFC is comprised of the agranular and anterior cingulate cortex[1], which receives projections from sensorimotor areas and forms strong reciprocal connections with dorsal thalamic nuclei[2]. The ventral portion of the mPFC is itself subdivided into the prelimbic (PrL) and infralimbic (IL) cortices. These areas send and receive projections from several subcortical brain regions that are primarily limbic in nature[2]. However, there is significant heterogeneity in their projection patterns and functional roles[3,4].

Recent data has revealed a complex functional interaction between the mPFC and components of the Hypothalamic-Pituitary-Adrenal (HPA) axis. Initial reports examining this interaction revealed that exposing mice to forced swim stress, a known potent activator of the HPA axis, significantly increased glucose metabolism throughout the mPFC[5]. A subsequent study corroborated this finding, showing that acute stress increased c-Fos expression, a marker of neuronal activation, throughout the mPFC[6].

Discrete subdivisions of the mPFC appear to have functionally distinct modulatory effects on the HPA axis. Lesions of the more dorsal aspects of the mPFC (anterior cingulate and PrL areas) robustly increased adrenocorticotropin hormone (ACTH) and corticosterone (CORT) secretion[7], and increased c-Fos expression in the paraventricular nucleus of the hypothalamus (PVN)[8]. Conversely, lesion of the infralimbic (IL) area of the mPFC both decreased the activity of CRH-secreting PVN neurons and attenuated HPA activation[9]. In this way, the PrL and IL cortical regions play largely opposing roles in regulation of HPA axis activity.

The antagonistic role of the PrL and IL cortices in regulating the stress response is partially explained by divergence in the regions to which they project. The IL cortex projects most strongly to brain regions involved in responding to acute presentations of fear and potentiating HPA axis activity, including the basomedial and central amygdala, the lateral septum, the anteroventral region of the bed nucleus of the stria terminalis (BNST), and the nucleus of the solitary tract[3,10]. In contrast, the PrL region projects more robustly to areas of the brain that exert an inhibitory influence over the HPA axis, including the paraventricular thalamus, basolateral amygdala, dorsomedial regions of the BNST, ventral subiculum, and a group of GABAergic cells that surrounds and innervates the PVN[3,10,11]. It is important to note that consensus on the exact

projection patterns of the PrL and IL cortices has not been fully reached. This lack of agreement is in part the result of discrepancies in defining the border between the PrL and IL regions, which is accomplished by establishing differences in projection patterns, cellular and genetic markers, and cytoarchitectural features[12].

The structure and function of the mPFC are dynamically altered in response to both acute and chronic stressors[13], including modifications to different neurotransmitter signaling systems and cytoarchitectural reorganization under certain conditions[14]. Overall, these changes cause a shift from a reflective to a reflexive brain state, resulting in the impairment of many PFC-mediated higher order cognitive functions[13].

Chronic stress induces robust morphological changes in the mPFC including retraction of dendrites and loss of spines from layer 2/3 (L2/3) pyramidal cells (PCs), as well as a loss of the apical tufts of layer 5 (L5) PCs[15,16,17]. These losses are coupled with enhanced dendritic outgrowth in limbic regions, such as the amygdala, further shifting the imbalance in favor of limbic nuclei[18]. Interestingly, this morphological adaptation is restricted to L2/3 neurons involved in cortico-cortical projections, and L2/3 PFC neurons that project to the BLA do not show the expected pattern of dendritic atrophy[16]. These morphological findings from animal studies are corroborated by human studies that show that chronic stress is correlated with a loss in cortical grey matter[13]. fMRI studies have revealed that subjects that have been exposed to chronic stress exhibit decreased functional connectivity in the mPFC, as well as decreased mPFC regulation of the amygdala[19,20].

The underlying changes in neurotransmission that drive these morphological adaptations have been extensively investigated. Under basal conditions limbic nuclei involved in emotional processing are under top-down control by projections from the PFC and phasic release of catecholamines in the PFC appears to enhance cognitive functioning, allowing for proper regulation of action, emotion, and thought[21]. However, stress exposure causes excessive release of both catecholamines and glucocorticoids in the mPFC. The end result is diminished ability of the PFC to exert top-down control over more 'primitive' structures, leading to an enhancement of conditional emotional responses and reflexive/habitual responses[13,21].

The ECS has been shown to play a key role in modulating the stress response in the mPFC[22]. Initial studies showed that stress exposure dramatically alters the levels of the two primary endocannabinoid ligands, 2-arachionoylglyerol (2-AG) and N-arachidonoylethanolamine (AEA), in multiple brain regions associated with stress and anxiety. This shift appears to be bidirectional, with stress decreasing AEA levels in the mPFC and amygdala while increasing 2-AG levels in the mPFC and hippocampus[23,24,25,26]. This data, along with hundreds of years of anecdotal reports of the stress alleviating and anxiolytic effects of cannabis (which activates the ECS) suggests that ECS could be an exciting target for novel therapeutics designed to treat stress and anxiety disorders[27,28,29].

Discovery of cannabinoid receptors

The study of the ECS took off in the early 1990s following the cloning and characterization of the Cannabinoid Receptor Type 1 (CB1) in 1991 and the Cannabinoid Receptor Type 2 (CB2) in 1993[30,31]. These receptors were both initially identified through their binding with Δ9-Tetrahydracannabinol (THC), the primary psychoactive constituent of marijuana[32]. These receptors have vastly different expression patterns, with the CB1 receptor being abundantly expressed in the central nervous system (CNS) and the CB2 receptor predominantly being localized to immune cells[30,32]. However, the precise expression pattern of CB2 in the CNS remains uncertain. Although studies have definitively shown expression of CB2 on glia, other studies have somewhat inconclusively suggested that CB2 is localized to neurons as well[33,34]. Here, neuronally-based CB1 and its signaling will be discussed; immune cell-based CB2 signaling is outside of the scope of this review.

CB1 receptors are primarily expressed on presynaptic neuronal elements[32], with the majority of histological studies pointing to higher expression at inhibitory rather than excitatory

synapses[35,36]. This localization ideally positions CB1 for modulation of neurotransmitter release[35].

Cannabinoid receptor signal transduction mechanisms

CB1 receptors are members of the G-protein coupled seven-transmembrane domain receptor superfamily. They primarily couple to Gi/o proteins, thereby inhibiting adenylyl cyclase, decreasing cyclic adenosine monophosphate (cAMP) levels, a ubiquitous second messenger associated with cellular activity, and ultimately decreasing cellular excitability. This decrease is blocked by pertussis toxin, confirming that this effect is mediated by a Gi/o dependent mechanism[37]. CB1 receptors are also capable of coupling to Gq proteins in vitro[38].

Moreover, CB1 has been shown to directly modulate ion channel conductance through interaction with the $G_{\beta\gamma}$ subunit. Activation of CB1 in vitro increases G-protein coupled inward rectifying potassium channel (GIRK) conductance, resulting in cellular hyperpolarization[39], and negatively regulates N and P/Q type calcium channels, leading to decreased presynaptic Ca^{2+} influx, decreased vesicle fusion, and decreased presynaptic neurotransmitter release[40,41,42]. A novel signal transduction cascade involving CB1 modulation of hyperpolarization-activated cyclic nucleotide-gated channels (HCN) via $G_{\beta\gamma}$ has recently been revealed to negatively regulate cellular excitability. Strangely, this phenomenon relies on activation of post-synaptic CB1 in a select subset of hippocampal Cornu Ammonis 1 (CA1) cells, which in turn activates a signal transduction cascade that increases HCN conductance, leading to a decrease in dendritic temporal summation and excitability and decreased probability of action potential firing[43].

Discovery, biosynthesis, and metabolism of cannabinoid receptor ligands

The discovery of the CB1 receptor spurred the search for its endogenous ligands. Within a year of cloning CB1, the endogenous ligand N-arachidonoylethanolamine (AEA) was isolated from the pig brain[44]. Less than three years later 2-arachidonylglycerol (2-AG) was isolated from the canine gut[45]. Pharmacological profiling of these two endogenous ligands revealed distinct receptor affinities. 2-AG acts as a full agonist at both CB1 and CB2, suggesting that it is a natural ligand for both receptors[46], while AEA acts only as a partial agonist. AEA is also an agonist at the transient receptor potential cation channel subfamily V member 1 (TRPV1)[46,47]. In addition to AEA and 2-AG, a number of other lipid signaling molecules have been documented to bind to the cannabinoid receptors with some affinity, although their role in inter and intracellular signaling is poorly understood[48,49,50].

Unlike conventional neurotransmitters, endocannabinoids are not packaged into vesicles at the synapse in preparation for release[32]. Instead, both AEA and 2-AG are synthesized on demand in response to cellular excitation[51,52] and a rise in intracellular Ca^{2+} levels[52,53,54]. However, they are formed by distinct biosynthetic pathways. AEA biosynthesis involves the transfer of arachidonic acid to the free amine of the membrane phospholibid phosphatidyl ethanolamine via the enzyme N-acetyltransferase. This results in the production of N-arachidonyl phosphatidylethanomaline (NAPE)[55,56], which can then be converted into AEA through a number of different biosynthetic pathways, including those catalyzed by phospholipase A2 (PLA2), phospholipase C (PLC), and NAPE-hydrolyzing phospholipase D (NAPE-PLD)[57]. The biosynthetic pathway for 2-AG appears to be somewhat more straightforward, with PLC hydrolyzing an arachindonic acid containing membrane phospholipid to produce diacylglycerol (DAG), which is then hydrolyzed by DAG lipase (DAGL) to produce 2-AG. Although alternate biosynthetic pathways have been proposed, this pathway accounts for the vast majority of 2-AG production[58].

Following synaptic release, endocannabinoids are quickly degraded by one of two major biochemical processes: hydrolysis or oxidation. Fatty acid amide hydrolase (FAAH), a post-synaptically localized enzyme, is responsible for the hydrolytic degredation of AEA22,[59]. 2-AG hydrolytic degradation is mediated primarily by the enzyme monoacylglyercol lipase (MAGL), and

partially by the serine hydrolases alpha/beta hydrolase domain containing 6 and 12 (ABHD6 and ABHD12)[60,61,62]. MAGL, ABHD6, and ABHD12 have different subcellular localizations, which may indicate access to and degradation of distinct pools of 2-AG content[61]. Interestingly, the oxidative degradation pathway appears to be partially shared between 2-AG and AEA. Both of these endocannabinoids are recognized and broken down by cyclooxygenase-2 (COX-2) in vitro[63], and can be oxidized by a myriad of lipoxygenase (LOX) enzymes[32]. However, these pathways are not as well understood as the canonical hydrolytic pathways, and require further investigation.

Endocannabinoids and synaptic plasticity

In the early 90's Llano et al.[64] observed the phenomenon of depolarization induced suppression of inhibition (DSI) in cerebellar slices, and proposed that this effect was mediated by a retrograde messenger. This DSI was characterized by a decrease in inhibitory postsynaptic current (IPSC) amplitude following positive voltage pulses in Purkinje neurons, and was dependent on postsynaptic Ca^{2+} influx[65]. Llano et al.[64] identified this as a presynaptic mechanism, as DSI decreased mini IPSC frequency.

In the early 2000s, a number of studies emerged espousing the endocannabinoids as such retrograde messengers[66,67,68]. In 2001, the Kano[69] and Nicoll[66] groups independently demonstrated that this form of short-term depression of inhibitory neurotransmission was dependent on CB1 signaling. A role for endocannabinoids in the depression of excitatory transmission through a process termed depolarization induced suppression of excitation (DSE) followed shortly thereafter[70]. Interestingly, 2-AG seems almost exclusively to mediate both of these processes of endocannabinoid mediated short-term depression[70].

Substantial effort has been aimed at elucidating the mechanisms that drive these forms of short-term depression. Three broadly defined categories through which 2-AG mobilizes to elicit this suppression of neurotransmission have been discovered: Ca^{2+}-driven endocannabinoid release (Ca^{2+}-ER), basal receptor-driven endocannabinoid release (basal RER), and Ca^{2+}-assisted receptor-driven endocannabinoid release (Ca^{2+}-assisted RER)[32].

Ca^{2+}-ER is the canonical form of endocannabinoid-mediated short-term depression[66,69]. Strong post-synaptic depolarization causes voltage gated calcium channels (VGCCs) to open, leading to a large increase in intracellular Ca^{2+} concentration[65] which in turn activates DAGL. Recent studies have suggested that full activation of DAGL is dependent on its interaction with membrane bound Homer proteins[32,71]. DAGL stimulates the production and release of 2-AG, which retrogradely travels to the presynaptic terminal to activate CB1 and suppress neurotransmitter release.

Basal RER was initially described after it was discovered that certain G_q-coupled receptor agonists induced a transient decrease in excitatory postsynaptic potential (EPSP) or inhibitory postsynaptic potential (IPSP) amplitude. Subsequent studies showed that this effect was 2-AG mediated and depended on G_q stimulation of PLCβ to produce DAG, which is then converted to 2-AG by DAGL[72].

Finally, Ca^{2+}-assisted RER represents a synergistic effect between Ca^{2+}-ER and basal RER. Weak postsynaptic depolarization delivered coincidently with a low concentration of a G_q-coupled receptor agonist can induce strong 2-AG mediated DSE or DSI that is dependent on activation of PLCβ and DAGL[36,73].

Endocannabinoids are also an important regulator of long-term plasticity. Studies have shown that in certain brain regions, such as the dorsal striatum, an endocannabinoid mediated form of long term depression (ECB-LTD) can be reliably invoked by both high and medium frequency stimulation protocols[74,75,76]. ECB-LTD appears to be primarily mediated through Ca^{2+}-assisted RER, as it is dependent on both activation of G_q-coupled receptors and Ca^{2+} influx through VGCCs[77,78]. However, there appears to be significant heterogeneity in the ability to induce ECB-LTD that is both brain region and cell type dependent[32,79].

Endocannabinoid signaling in the prefrontal cortex

As previously mentioned, the PFC expresses all of the major components of the ECS and appears to be a major site of action for the anxiolytic properties of cannabinoid agonists such as THC[80,81]. CB1 has a distinct laminar distribution pattern in the PFC, with the strongest density of CB1 located on GABAergic cells in L2/3 and sparser expression in L5[82]. This is closely paralleled by physiological data showing that the vast majority of L2/3 cells exhibit DSI, which in the PFC can be induced both by electrical depolarization as well as activation of G_q receptors such as mGluR5[83], while only a small proportion of L5 cells do[84]. Notably, CB1 expression appears to be significantly higher in affective association and cognitive processing areas of the cortex, such as the anterior cingulate and ventral mPFC, than in motor and sensory areas[85].

Early immunohistochemistry (IHC) studies suggested that CB1 was solely expressed on inhibitory interneurons in the PFC. The development of more selective antibodies and improvements in electron microscopy have since led to the discovery of robust CB1 expression on glutamatergic axon terminals in the PFC[86]. This finding is supported by physiological data demonstrating that 2-AG dependent DSE can be readily induced in L5 projections neurons[87]. Additionally, endocannabinoids mediate muscarinic acetylcholine receptor (mAChR)-dependent LTD in L5 PCs, suggesting that endocannabinoids are signaling in a retrograde manner to dampen excitatory input onto these cells[88]. These studies and others suggest that endocannabinoids play an important role in gating excitatory input to the PFC.

Prefrontal cortical endocannabinoid regulation of stress and affective behaviors

CB1 receptors on glutamatergic terminals and GABAergic terminals in the PFC play distinct roles in regulating different affective behavioral states. For example, disinhibition of L5 PCs by GABAergic CB1 appears to play a causal role in persistent pain-induced impairments in cognitive flexibility[89], as well as chronic stress-induced cognitive deficits[90]. Conversely, the expression of CB1 receptors on glutamatergic neurons in the mPFC appears to be important for both neuroprotection from excitotoxic seizures as well as appropriate neuroendocrine and behavioral responses to stress[22,91,92]. Furthermore, these two different CB1 receptor populations appear to underlie the dosage dependent psychoactive effects of THC, with glutamatergic CB1 mPFC receptors mediating the anxiolytic effects of low dose cannabinoids and GABAergic CB1 mPFC receptor activation medating the anxiogenic effects elicited by high doses of THC[93].

Data from behavioral and morphological analyses of CB1 knockout (KO) mice supports a recent hypothesis that PFC CB1 could play an important role in regulating the neuroendocrine response to stress. Under basal conditions CB1 KO mice display a chronic stress-like behavioral state[90] and a robust atrophy of L2/3 dendritic arbors, which closely parallels the morphological changes induced by chronic stress in rodents[90]. Additionally, CB1 KO mice show heightened levels of corticosterone secretion in response to acute restraint stress, suggesting that these mice have an impaired ability to regulate neuroendocrine responses to stress[94,95].

Recently, there has been increased interest in understanding how neuroendocrines influence cannabinoid signaling in the mPFC. Work by Hill et al[22]. has demonstrated elevated levels of 2-AG in the mPFC 60 minutes after exposure to acute restraint stress. This increase appears to be dependent on the activation of glucocorticoid receptors by circulating corticosterone[26]. Additionally, inhibition of CB1 in the PrL cortex following restraint stress significantly prolonged the time course of corticosterone secretion, suggesting that this population of CB1 receptors is involved in feedback inhibition of the HPA axis[26]. This hypothesis is supported by data showing that application of corticosterone to mPFC slices decreases GABA release onto L5 projection neurons[22]. Together, these studies suggest that this feedback mechanism is activated by glucocorticoid mobilization of endocannabinoids in the mPFC, leading to disinhibition of PFC output to subcortical areas involved in negative regulation of the neuroendocrine stress response[26]. This increase in 2-AG content in the mPFC following chronic stress may be critical for habituation to initially stressful stimuli[80].

Stress appears to have the opposite effect on AEA levels in the mPFC. Acute stress causes a rapid decrease in AEA content in the PrL cortex that appears to be caused by increased FAAH activity[96]. Correspondingly, local inhibition of FAAH within the PrL cortex decreases stress-induced corticosterone secretion[22]. Together these results point to a differential role for 2-AG and AEA in modulating PFC-regulation of the neuroendocrine stress response.

Autoradiographic and IHC studies have demonstrated that different stress paradigms robustly alter CB1 expression and binding density in the PFC. Chronic unpredictable stress (CUS) and restraint stress lasting both 4 and 10 days increases CB1 expression in the PFC[97,98,99]. Interestingly, inhibition of FAAH in the PFC can reverse this increase in CB1 expression, suggesting CB1 expression is initially enhancedto compensate for the decrease in AEA levels following stress[22,99].

Despite data linking the ECS to the regulation of the neuroendocrine stress response, it is important to understand that the correlation between neuroendocrine activation and the emotional response to stressful situations is not exact. Stress is the response of an organism to external stimuli that threatens well-being or homeostasis. Emotionality, on the other hand, encapsulates the organism's subjective experience, which is often accompanied by behavioral or physiological reactions to the environment. Nonetheless, stress is a ubiquitous risk factor for the development of negative emotional states and neuropsychiatric disorders such as anxiety, depression, and post-traumatic stress syndrome (PTSD)[22].

Therapeutic potential of the endocannabinoid system

Emerging evidence suggests that local manipulation of the ECS in the mPFC could serve as a novel therapeutic target for the treatment of affective disorders. For example, local administration of cannabinoid agonists to the mPFC elicits a potent anxiolytic effect[81], while overexpression of FAAH in the mPFC induces anxiogenic effects, reinforcing the idea that the stress-induced decrease in cortical AEA could precipitate anxiety disorders[81]. Furthermore, inhibition of anandamide hydrolysis in the ventromedial mPFC produces an anti-depressant effect in the forced-swim test[96]. Of note, a similar anti-depressant effect is elicited by deep brain stimulation of the analogous brain region in humans, and there is evidence to suggest that this effect is dependent on AEA's ability to stimulate serotonergic signaling[100,101,102].

As previously discussed, a substantial amount of evidence has revealed that certain components of the ECS are upregulated in the mPFC following stress exposure, including 2-AG and CB1 expression. This increase in CB1 expression is paralleled in post mortem analyses of suicide victims and patients who suffered from major depressive disorder[103]. The initial hypothesis was that increased cannabinoid signaling represented a detrimental maladaptive consequence of life stress. However, an alternate explanation is that the ECS was recruited in the mPFC to dampen the negative physiological effects of stress. In line with this hypothesis, McGlaughlin et al.[104] showed that the upregulation of CB1 in the mPFC following stress is a positive adaptive response that represents a neural coping strategy against stress. Follow-up studies corroborated this compensatory role for the ECS in the mPFC in opposing the maladaptive changes induced by stress. Treatment with a CB1 agonist dampens a wide variety of neuroinflammatory and excitotoxic effects elicited by chronic stress[97]. Furthermore, decreasing endocannabinoid signaling in the mPFC elicits similar morphological changes to dendritic structure as chronic stress[90]. These data strongly suggest that endocannanbinoid signaling in the mPFC functions as a buffer against the negative physiological consequences of stress exposure.

Proposed model linking the prefrontal cortical endocannabinoid system and stress

Given what we know about the effects of stress on the PFC and the ECS, we propose the following model for the role of prefrontal endocannabinoids as a buffer against stress induced affective disturbances: in a stress-free basal state, there are high levels of tonic AEA release in

the PFC, which functions to constrain glutamatergic drive from limbic brain regions, such as the BLA and ventral hippocampus (vHIP) (Figure 1A)[22]. Following stress exposure, FAAH activity in the PFC is decreased, reducing inhibitory tone on glutamatergic projections to the PFC[96]. Furthermore, stress has been shown to increase the magnitude of excitatory input from several limbic brain regions to the PFC[14,105,106], and increased excitatory neurotransmission within the mPFC is associated with stress susceptibility (Figure 1B)[107]. Shortly after stress onset, circulating corticosterone binds glucocorticoid receptors in the PFC. This binding leads to increased production of 2-AG[26], which is subsequently released from the post-synaptic cell, binds CB1 on glutamatergic terminals arriving from limbic brain regions, and decreases excitatory glutamatergic drive onto the mPFC (Figure 1C). Decreasing excitatory input to the mPFC has been shown to promote resiliency to stress[107].

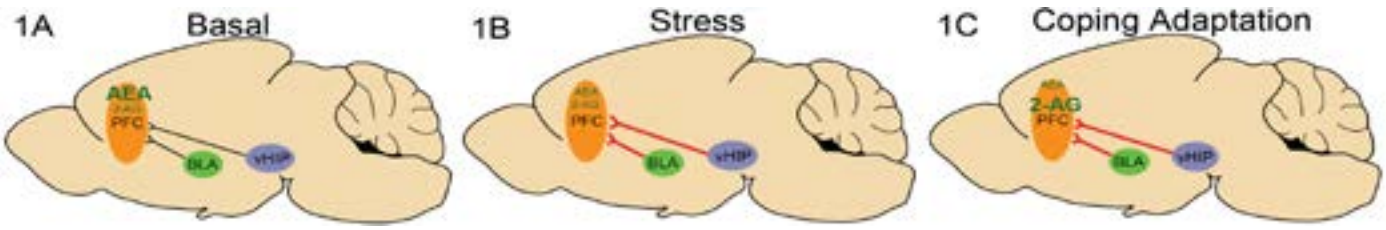

Figure 1. The ECS as a potential buffering system in the mPFC following stress. A) Under basal conditions, AEA tonically inhibits glutamatergic drive from limbic brain regions. B) Stress increases the glutamatergic drive from limbic brain regions to the mPFC, and decreases AEA levels in the mPFC. C) Following stress exposure, gluocorticoids stimulates 2-AG production, leading to activation of CB1 on glutamateric terminals in the mPFC and ultimately resulting in decreased magnitude of excitatory input.

Future Directions

Although data for the role of the mPFC ECS in buffering stress-induced neurophysiological changes is strong, the neural circuits underlying these cannabinoid mediated effects remain unknown. The development of the optogenetic toolbox over the past decade has opened up the possibility of functionally interrogating intact neural circuits[108]. Distinct projections to the PFC are causally implicated in a wide variety of affective behaviors and motivational states[4,13,109,110]. A better understanding of how stress is able to selectively augment endocannabinoid signaling in distinct projections to the mPFC will provide us with greater insight into the mechanisms behind the anxiolytic and stress attenuating properties of exogenous cannabinoids and possibly spur the development of novel cannabinergic therapeutic compounds.

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Multisensory Spatio-Temporal Interactions in Depth: the Boundaries of Integration

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Abstract

The study of how distinct sensory modalities are integrated in the brain in order to paint a cohesive multisensory representation has established a set of governing rules that enhance the likelihood of sensory binding. Among others, these principles, established first by single-cell recording in animal models, but also applicable to indices of larger neural ensembles in humans, state that the closer in time or space two unisensory stimuli are from one another, the more likely they will be integrated. Although these principles are well characterized, a surprising lack of evidence indicates how they interact with one another – just as they would in the real world, stimuli features change dynamically and non-independently as they move through space. Here, I propose that two recent notions within the psychophysical study of multisensory interactions may be amenable to the study of not only multisensory, but also spatio-temporal integration. The temporal binding window (TBW – the temporal disparity over which distinct unisensory stimuli are likely to be characterized as a synchronous multisensory event), and the delineation of peri-personal space representations (PPS – the proximo-distal spatial disparity over which exteroceptive sensory signals influence somatosensory processing) are both multisensory indices of the boundaries of integration. Hence, in the following review, I highlight the need to examine spatio-temporal multisensory interactions. In turn, I examine literature pertinent to the TBW and PPS, and finalize by underlying studies examining temporal and spatial aspects of audio-visual integration. I suggest that these latter studies provide a springboard for the examination of multisensory spatio-temporal interactions.

Introduction: Multisensory Principles

Our world is made up of a wealth of information from a number of distinct sensory modalities – a multisensory mélange that we seamlessly integrate into a coherent perceptual unity. Whereas visual, auditory, and tactile information are initially processed independently, the successful integration across these systems is a critical aspect of our behavioral repertoire facilitating and enriching a host of perceptual tasks and experiences.

At the neurophysiological level, much work has gone into characterizing the response properties and profiles of multisensory neurons (particularly in the superior colliculus) when presented with stimuli from multiple sensory modalities. These seminal studies revealed striking non-linearities (e.g., supra- and sub-additivity with regard to statistical summation) in the multisensory responses of these neurons[1-8], and have elucidated a set of integrative "principles" by which these neurons operate[9,10]. These principles revolve around the statistical features of the stimuli that are to be combined, including their spatial and temporal relationships to one another, as well as their relative effectiveness. In the simplest terms, these principles state that stimuli that are spatially and temporally proximate and weakly effective (when presented on their own) give rise to the largest proportionate enhancements of response when combined. As stimuli are separated in space and/or in time, and as they become increasingly effective, these proportionate multisensory gains decline[11,12].

Although first established at the level of the single neuron in animal models, these principles have equally been shown to apply to indices of activity in larger neuronal ensembles such as scalp[13] and intracranial EEG[14], as well as fMRI[15], and indices of animal and human behavior. Examples of multisensory-mediated benefits in the behavioral and perceptual realms include enhanced target detection[16,17], facilitated target localization[18,19], increased intelligibility of spoken signals[20] and speeded reaction times[21-23] in response to spatiotemporal-proximate and weak stimulus pairings (although see[24] and[25] for relevant discussions on the applicability of the so-called principles of multisensory integration to neuroimaging indices in humans).

Integrating Principles: Lack of a Spatio-Temporal Understanding

Despite the large and rapidly growing number of studies detailing how stimulus factors shape multisensory interactions, the vast majority of previous reports have looked at each of these factors in isolation, or have employed a set of features (e.g., temporal processing) in order to examine the other (e.g., spatial), but have not systematically examined their interaction. For example, the majority of studies structured to examine the impact of spatial location and correspondence between stimuli have only manipulated stimuli in the spatial dimension(s) while presenting stimuli synchronously. Similarly, temporal factors such as the temporal discrepancy between consecutively presented stimuli have routinely been employed in order to measure spatial aspects of multisensory interactions, without systematically manipulating both spatial and temporal aspects together (e.g., [26]; see [27], for review). In addition, temporal factors have been regularly employed not only to measure, but also to manipulate spatial multisensory correspondences (e.g., synchronous administration of visuo-tactile stimulation on a rubber hand in order to elicit an illusory feeling of body ownership), nonetheless these studies structured to examine the temporal dynamics underlying multisensory interactions have typically done so at a single (or a very restricted set of) spatial location(s). Although informative, it must be pointed out that such manipulations typically differ from real environmental circumstances, where dynamic stimuli are changing frequently and non-independently in effectiveness and in their spatial and temporal relationships.

Some recent studies have begun to examine the interactions between space and time in the processing of multisensory information at the level of the individual neuron, giving rise to the notion of multisensory spatio-temporal receptive fields[28,29]. These studies, however, have remained inconclusive (e.g., simply revealing heterogeneity) and have remained purely descriptive. Even fewer reports have systematically examined these issues in the domain of human performance (although see [30-32] for exceptions). In addition, those few studies that have attempted to examine multisensory spatio-temporal interactions have almost exclusively done so in azimuth and elevation, rather than depth, and have revealed equivocal results. For example, examination of the effects of spatial alignment on temporal perception have shown cases of benefit, other cases of a deterioration in performance, and still others with no obvious interaction[30,33-36].

Here, I propose that in recent years two parallel notions have been respectively raised within the psychophysical study of multisensory interactions across time and space, which may be particularly amenable to elucidating the principles guiding not only multisensory, but also spatio-temporal integration. These are the concepts of the multisensory temporal binding window (TBW; the temporal disparity between unisensory signals that participants tolerate while still considering these as co-occurring in time, and thus as putatively emanating from a single multisensory source; See Figure 1A) and the delineation of peri-personal space boundaries (PPS; the proximal-distal space over which exteroceptive sensory signals facilitate processing of somatosensory information on the body; See Figure 1B). These two concepts therefore refer to the (temporal and spatial) boundaries over which multisensory interactions are likely to occur. In the following two sections, hence, I review in turn the literature pertaining to the TBW and PPS, while attempting to highlight similarities and dissimilarities between these fields of study. Lastly, I finalize by underlying that by and large studies aimed at scrutinizing the temporal factors governing multisensory processing (e.g., TBW) have employed audio-visual stimuli, while those utilized in the demarcation of PPS boundaries have employed audio- and visuo-tactile. The discrepancy in sensory modalities traditionally studied in the demarcation of the temporal and spatial multisensory boundaries of integration thus hinder the opportunity to reveal commonalities and mechanistic principles governing the delineation of spatio-temporal multisensory processing limits. I conclude that in order to determine the interdependence between temporal and spatial boundaries of multisensory processing, we must begin examining audio-visual interactions in time and space as a function of the distance between the observer and the stimuli presented; that is, as spatio-temporal relations are moved from peri- to extra-personal space.

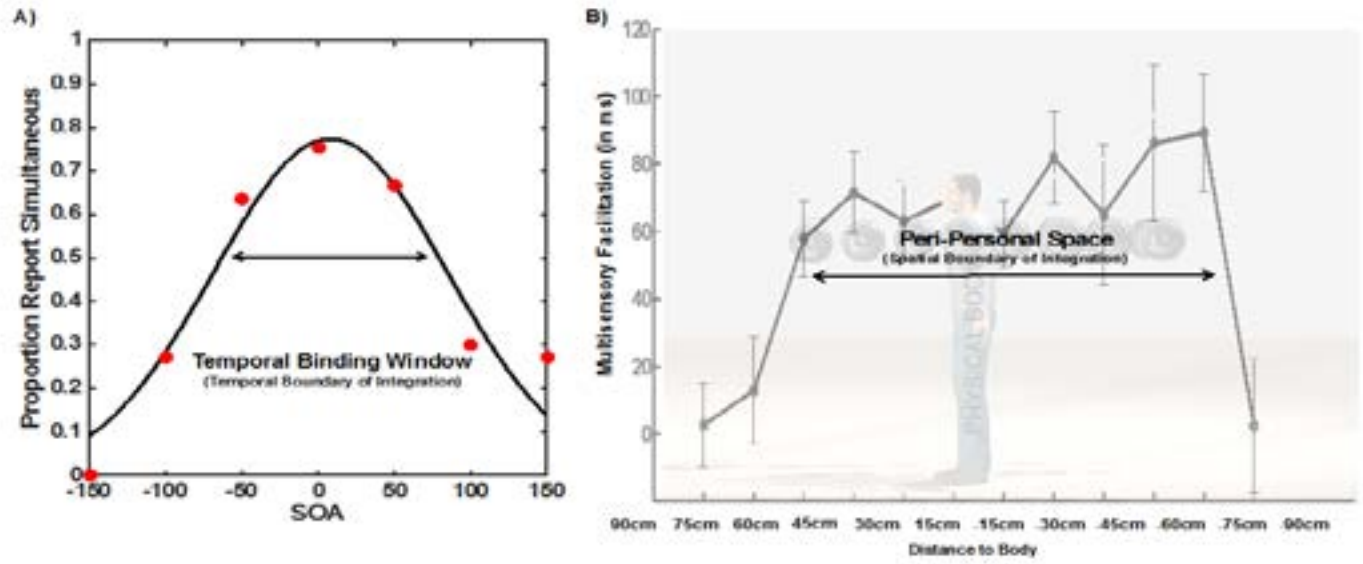

Figure 1. Temporal and Spatial Boundaries of Multisensory Integration. A) Simultaneity judgment tasks in which participants report whether two unisensory stimuli presented at a given stimuli onset asynchrony (SOA, x-axis) were presented synchronously or not (y-axis) have been extensively utilized in the past in order to determine the temporal boundary of integration. B) On the other hand, speeded multisensory (vs. unisensory) reaction times (y-axis) as a function of stimuli distance (x-axis) have been routinely employed in order to scrutinize the spatial boundary of multisensory interactions. Figure (part b) adapted from Noel et al., 2015.

Temporal Features of Multisensory Processes: the Temporal Binding Window

In the temporal domain, in addition to emphasizing the importance of the stimulus onset asynchrony (SOA) at which stimuli occurred in determining the outcome of a given multisensory pairing, experiments in the superior colliculus of the cat showed that the span of time over which response enhancements are generally seen in these neurons is on the order of several hundreds of milliseconds[5,7,37,38]. Behavioral studies have followed up on these analyses to illustrate the temporal constraints of multisensory combinations on human performance, and have found that the presentation of multisensory stimuli either synchronously or in close temporal proximity results in shortened saccadic and manual reaction times[23,39], heightened accuracy in understanding speech in noise[40-42,] and play an important role in an array of multisensory illusions, such as the sound-induced flash illusion[43,44] or the rubber-hand illusion[45,46]. Perhaps the most striking feature of multisensory temporal processing is the large extent of time over which stimuli presented in distinct sensory modalities may influence one another. This property, the TBW, makes good ethological sense, and is seemingly adaptive, in that it provides a buffer for the latency differences that characterize the propagation times, as well as the neural transduction times, of energies in the different senses.

The postulation of these TBWs as adaptive equally emerges from a panoply of studies demonstrating that these windows of multisensory binding are both plastic and protracted in development, allowing them to accurately reflect the sensory statistic of their environmental surrounding. Stevenson and colleagues[47-49] have characterized the size of TBWs over an array of sensory stimuli; most notably, simple flash and beeps, complex non-speech stimuli, and speech stimuli (i.e., utterance of a single syllable). The results of these studies have routinely indicated that TBWs are larger for the most complex stimuli, in particular speech, giving rise to the interpretation that a learned tolerance for asynchrony is greatest for stimuli to which we are most exposed[50]. These windows are equally plastic in that after repeated exposure to a certain asynchrony, participants' reports[51,52] and animal neural firing rate[53] are maximal to the new most common sensory statistic. These recalibration effects have been utilized successfully in multisensory training paradigms[54], and though they were initially demonstrated after a prolonged exposure to asynchronies, recent work has highlighted that TBWs will reflect the statistics of the world even on a trial-per-trial basis. That is, if for example, on a given trial an auditory-leading stimulus is presented,

the distribution of responses for the next trial will be biased toward indicating auditory-lead on the subsequent trial[55-59].

Similarly, developmental studies in both animal models[37,38,60-62], and humans[63-65] have indicated that TBWs do not reach a mature-like state – i.e., smaller in size – until late adolescence[59,66,67]. Further, the maturational processes, as well as the adaption effects described above, are dependent on the sensory pairing to be combined[5,68-70], but see [59]). Indeed, pairing with the tactile domain – audio-tactile or visuo-tactile – become mature before the audio-visual pairing. Seemingly, the delayed and pairing-specific timecourse of development in establishing TBWs permits an accurate representation of the sensory asynchronies present in our environmental surrounding.

Spatial Features of Multisensory Processes: Peri-personal Space Representation

An analogous concept to the TBW in the spatial dimension is, from a neurophysiological perspective, the notion of receptive fields, and from a psychophysical perspective, arguably the concept of peri-personal space (PPS). PPS – the space immediately adjacent to and surrounding your body[71-73] – the bubble of space surrounding your body - was first described by neurophysiological recordings in non-human primates. Seminal studies described multisensory neurons in ventral intraparietal (VIP) and ventral premotor cortex that responded most notably to visuo-tactile but also audio-tactile stimuli that were presented close to but not far from the body[74-79]. These neurons are anchored on specific body parts – the hand, face, and trunk – as their receptive fields are not retino-centered, but moved with the body[79,80]. The observation that these neurons were equally motor, in that an electrical impulse would evoke a stereotyped defensive-like movement[81,82] and that they responded most vigorously to dynamic looming stimuli[83], elicited the postulation that this fronto-parietal neural network is involved in defensive and obstacle avoidance behavior (for review see [84]).

A corresponding motor-multisensory spatial boundary has been characterized in humans both from a neuroimaging[85-88] and behavioral[89-92] perspective (for recent reviews see [93,94]). As for the TBW, it is postulated that this window over which visuo-tactile and audio-tactile interactions occur is adaptive in that it is plastic and reflects the natural sensory statistics of the world. The size of PPS representation in humans has been demonstrated to adjust based on motor expertise[95-97] (see [98], for a similar finding in monkeys) and execution[86,92,99,100], individual socio-personal states and traits[101-104], and stimuli valence[105] (see [106-108] for reviews).

Thus, as in the study of the limits of multisensory temporal processing, findings with regard the spatial nature of multisensory binding – at least regarding the study of PPS – have indicated i) relatively large windows of integration, which are ii) incredibly flexible to both stimuli- and individual-driven features, seemingly conferring an adaptive advantage in that these windows reflect and adjust to the natural statistics of the environment. However, as exemplified by the array of reports mentioned above, the study of the temporal limits of multisensory integration (e.g., TBW), and the scrutiny of the spatial boundaries of multisensory binding (particularly in the proximo-distal spatial dimension) have heavily focused on distinct sensory pairings. Namely, the study of the temporal aspects governing multisensory integration has focused on audio-visual stimuli, while the spatial delineation of the boundaries of integration, in particular in the proximo-distal spatial dimension, has focused on the visuo-tactile and audio-tactile pairings.

Toward an Integrated Spatio-Temporal Characterization of Multisensory Processes

A question that remains open is how do these temporal and spatial binding windows relate to one another? The demarcation of TBWs has focused heavily on the audio-visual pairing, though researchers have also established audio-tactile, and visuo-tactile TBWs[92,109]. Further, the size of these temporal windows appear to be correlated

across sensory pairings and within individuals[70,110]. The study of PPS on the other hand, has focused quasi-exclusively on visuo-tactile, and to a lesser extent, audio-tactile interactions. This focus on somatosensation is for good reason, as PPS can be conceived as a stochastic sphere surrounding the body (or different body-parts) computing the probability with which different objects may come in contact with the body. Thus, a tactile component is required for a PPS representation[80,111]. Now, in order to study spatio-temporal interactions, and the impact of the spatial principle of multisensory integration in different depth planes (as done in PPS studies), audio-visual interactions must be examined. Indeed, a confound in all PPS experiments is that as either audio or visual stimuli are moved farther from participants, so is the distance between the corresponding unisensory components of the multisensory unit.

Studies have indeed examined aspects of multisensory spatial processing not involving touch (and thus not happening by necessity on the body). For example, in the case of audio-visual interactions, the spatial ventriloquism illusion[112] or audio-visual localization experiments (e.g., [113]) are well established. However, the vast majority of these studies have emphasized the notions of 'precision' or 'optimality', as opposed to scrutinizing the spatial and/or temporal breaking point of integration. Further, audio-visual spatial tasks have been carried out almost exclusively in elevation or azimuth, as opposed to depth (see [114]). Lastly, from a spatio-temporal perspective, studies have simply characterized which modality dominates under certain conditions. The modality appropriateness hypothesis[115], for instance, has emphasized that the modality conveying the most reliable/precise information will weigh more heavily in the computation at hand, and has pointed out that for temporal tasks, audition is most precise, while for spatial tasks vision dominates as visual spatial acuity is greater than auditory[116-119]. These studies, therefore, have emphasized relative weighting between sensory modalities, but have failed to demonstrate (or examine) spatio-temporal interactions. In the following sections, I review literature pertaining to audio-visual spatial and temporal interactions at different depth planes, thus providing the foundation for the putative delineation of not only temporal or spatial but also spatio-temporal binding windows.

Audio-Visual Interactions in Depth: Space

Besides the discrepancy in physical propagation times between audition and vision, other factors may play a role in altering audiovisual interactions as these stimuli are moved in the proximo-distal space[120]. For instance, auditory and visual information may be more dominant in far space, while tactile, nociceptive, proprioceptive, and intereoceptive signals may dominate in the close space. In addition, information available in far space is often used for orienting and navigation[121]. As a result, audiovisual integration may be expected to be greater at farther rather than closer distances. In fact, Van der Stoep et al.[122] recently observed that audiovisual integration in far space (200 cm), as opposed to near space (80 cm), was enhanced. This facilitation was evident from increased multisensory response enhancement (faster responses to multisensory targets relative to the fastest response to unisensory targets), and an increased amount of race model inequality violation in far space relative to near space[123-125] (see [126], for a similar result in a visual search paradigm). Importantly, the greater magnitude of multisensory integration could not be explained simply by a change in stimulus efficacy, as the same decrease in the size of the retinal image and intensity in near space as in far space did not give rise to enhanced multisensory integration. The effect could also not solely be explained based on the region of space in which the stimuli were presented (i.e., audiovisual dominance in far space), as there was no difference in integration between the near and far space condition when the stimuli were corrected for retinal image size and intensity. Thus, the authors concluded that both a decrease in retinal image size and intensity, and the region of space in which information was presented contributed to the observed enhancement of audiovisual integration. This study thus represents an elegant demonstration of the necessity to study principles of multisensory integration (in this case, the spatial and the inverse effectiveness rule) in conjunction with one another.

On the other hand, there is also support for the lack of such distance-specific enhancements of audiovisual spatial interactions. In a study of cross-modal spatial attention across and within different depth-planes, a cross-modal cuing effect was found to be dependent on whether the auditory cue and the visual target were presented in the same depth-plane[127]. However, the cuing effect within planes (e.g., audio and visual stimuli presented either near or far) did not differ. These findings, therefore, indicate that the strength of audiovisual interactions may not only be affected by whether spatially aligned multisensory stimuli are presented in near or far space (as seemingly indicated in [122], but not [127]), but also by whether the component auditory and visual stimuli are themselves presented at the same distance. Thus, the region of space in which information is presented may not only modulate multisensory interactions involving touch, but also audiovisual interactions[122], though this evidence is still only emerging.

Audio-Visual Interactions in Depth: Time

Given that the temporal relation between auditory and visual information depends on the distance from which the stimuli are presented, any resulting differences in arrival times may influence the strength of audiovisual interactions between stimuli presented from different distances. Indeed, Sugita and Suzuki[128] investigated whether the point of subjective simultaneity for visual and auditory stimuli depended on the perceived depth of the visual stimulus (see also [129]). Participants were explicitly instructed to imagine that the visual stimulus was the source of both the light and the sound (which was presented by means of headphones – and thus not co-localized with the visual stimuli, but always emanating from a source near the participant). As expected based on the differential propagation times of sound and light in space, increasing the distance of the visual stimulus increased the auditory delay that was necessary for subjective simultaneity for stimuli that were presented from distances up to 20 m. This study, thus, demonstrates that participants are able to take into account proximo-distal distance when performing audio-visual simultaneity judgments. However, it equally confounds the distance from the observer with the distance between the unisensory components of the multisensory unit.

More recently, Noel et al.[130], had participants perform an audio-visual simultaneity judgment while participants stood and the multisensory stimuli were co-localized and placed at 1 m from their eyes and ears. Importantly, the multisensory stimuli were either placed toward the top (and hence 1 m away from participant's eyes and ears, but also ~1 m away from the closest point of contact to the participant's body) or toward the bottom (and hence 1 m away from the participant's eyes and ears, but in this case only ~30 cm away from the closest point of contact to the participant's body). The 1 m condition was taken as being outside PPS, while the 30 cm condition was taken to be inside PPS[73]. By such manipulation, the researchers effectively manipulated the distance to the body from which audio-visual stimuli were presented, but kept lower-level sensory statistics (e.g., intensity, size) constant. The results demonstrated that audio-visual TBWs were larger inside as opposed to outside PPS.

These results at first pass may seem contrary to the above-mentioned demonstration that race-model violations are most prominent in far space than in near space[122]. However, race-model violations can be conceived as an index of multisensory integration 'magnitude' (i.e., degree of facilitation) while the delineation of TBWs is nothing but the demarcation of the temporal extent over which multisensory integration is likely. Thus, it may very well be that the degree to which multisensory integration occurs, and the temporal extent over which it occurs are modulated in different directions as stimuli are moved along the proximo-distal axis. A speculation that remains to be tested, nonetheless, one that is in line with the argument that the principles and classical demonstrations of multisensory integration (i.e., spatial and temporal rule, inverse effectiveness, race-model violations) must be studied in conjunction, and may demonstrate differential effects as stimuli are moved in three-dimensional space. Such demonstrations promise to pave the way for a more ecologically valid understanding of how multisensory stimuli are processed in the real world, where stimuli features change dynamically and non-independently.

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Evidence of Functional Dopaminergic Signaling in Mammalian Visual Cortex

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Abstract

Dopamine is a catecholaminergic neurotransmitter found in the mammalian visual cortex. However, the exact role of dopamine and the mechanisms by which dopamine influences neural responses within visual cortex remain unclear. In order to better understand the role of dopamine, the anatomy of the dopaminergic system within visual cortex must be well established. Model organisms typically used to study visual function (i.e. rats, cats, and monkeys) exhibit large interspecies differences in dopaminergic anatomy. Therefore, the predictive value of data collected from these model organisms should be carefully evaluated before applied to theories of human vision. Despite differences in dopaminergic anatomy, model organisms will continue to be critical to the functional evaluation of dopamine within visual cortex. This review discusses the dopaminergic innervation, concentrations, and receptor distributions within the visual cortex of several mammalian species. In addition, this review highlights studies that have demonstrated functional dopaminergic signaling within visual cortex. Finally, current theories concerning cortical dopamine will be touched upon, and potential future experimentation will be considered.

Introduction

Dopamine is a catecholaminergic neurotransmitter found in the mammalian nervous system. Dr. Arvid Carlsson discovered dopamine in 1952, and since its discovery, the role of dopamine in the brain has been extensively studied. Dopamine has been portrayed by media outlets as the brain's "reward" chemical, and is indeed a pivotal signaling molecule in the midbrain reward pathway. However, this view of dopamine is a gross oversimplification. In addition to reward, dopamine has been shown to help regulate critical functions such as hippocampal-mediated learning, emotional perception, and motor control[1–3]. Furthermore, irregularities in the dopaminergic system are believed to be involved in neuropathological diseases such as schizophrenia, clinical depression, and Parkinson's disease[4–6]. Dopaminergic nuclei in the midbrain project throughout the entire cortical mantle, yet how dopamine influences cortical circuits remains unclear. While some research has started to reveal the function of dopamine in frontal cortices[7–9], the role of dopamine in the sensory cortices remains relatively understudied. Of all the sensory systems, dopamine has been implicated in vision the least. However, there is clear anatomical evidence that dopamine has a functional role in processing visual information in the mammalian visual cortex.

The purpose of this review is threefold: **1.** Provide evidence that dopamine has a functional role in the processing of visual information in the mammalian brain. **2.** Highlight species differences in the dopaminergic innervation of the mammalian visual cortex. **3.** Speculate upon the source of dopamine in the visual cortex of the mammalian brain, and briefly propose future experiments that will help elucidate the role of dopamine in the visual cortex.

To more clearly address the objectives of this paper, it is important to briefly describe the signaling properties of dopamine. Within cortex, dopamine is believed to act as a neuromodulator. A neuromodulator is a chemical signal that regulates the excitability of neurons and alters the signal transduction of other neurotransmitters. Like other neuromodulators, dopamine often acts through volume transmission, diffusely affecting the perisynaptic space surrounding a release site[10]. This enables dopaminergic nuclei to affect large areas of cortex, but inherently constrains the involved circuits to function on slower temporal scales. The cortex uses a precise distribution of dopaminergic receptors to achieve spatial specificity from a diffuse signal. There are five types of G protein-coupled dopamine receptors (D1, D2, D3, D4, and D5); all of them utilize cyclic adenosine monophosphate (cAMP) as a secondary messenger. Dopamine receptors are divided

into two categories. D1 and D5 dopaminergic receptors are considered D1-like receptors, and D2, D3, and D4 dopamine receptors are considered D2-like receptors. The activation of dopamine receptors modulates cellular functions such as cell excitability, neurotransmitter synthesis, vesicular packaging, and membrane receptor expression[11].

The basic anatomy of the dopaminergic system within visual cortex is conserved across the mammalian linage. Midbrain dopaminergic nuclei send projections into visual cortex; the visual cortex, in turn, receives the signal in spatially discrete zones defined by unique distributions of dopamine receptors[12–15]. Despite this shared basic organization, the dopaminergic system within visual cortex varies drastically across species. First, the visual cortices of different species receive varying degrees of midbrain dopaminergic innervation. Second, dopaminergic projections terminate in different layers of visual cortex depending on species. Third, there are species differences in the distribution of dopaminergic receptors across the six layers of visual cortex. These differences are key to understanding how each organism utilizes dopamine to modulate visual processing. Furthermore, acknowledgment of these differences will help more accurately relate findings from studies on non-human species to the human visual system. Finally, relating species differences to phylogenic maps yields insight into the evolutionary changes that the visual dopaminergic system has endured.

Evidence from Rat

Early studies involving rodents provided the initial insights into the origin and regional specificity of cortical dopamine in the mammalian brain[16–18]. Radioautographic[a], immunohistological[b], and retrograde tracer[c] studies have revealed that the rat visual cortex (Brodmann's area 17) and extrastriate cortex (Brodmann's area 18a) receive sparse dopaminergic innervation compared to other cortical areas such as the frontal cortices. The rat visual cortex contains few dopaminergic terminals with most dopamine-immunoreactive fibers being secluded to layers V and VI[19,20]. The extrastriate cortex has a denser population of the dopaminergic varicosities, and dopaminergic axons can be found throughout all 6 cortical layers[21,22]. The primary source of dopaminergic innervation to rat visual cortex seems to originate in the rostral ventral tegmental area (VTA), but the substantia nigra pars compacta (SNpc) also appears to send a small number of projections to visual cortex[20,23].

Dopamine has been detected in rat visual cortex. High performance liquid chromatography (HPLC)[d] analysis has shown that homogenates derived from rat visual cortex contain dopamine at concentrations between 10-14ng/g[20]. An electrochemical analysis of extracellular dopamine collected via microdialysis has shown that rat visual cortex contains dopamine at a concentration of approximately 6ng/g[24]. Another study quantified the concentration of dopamine in the rat visual cortex using an enzyme isotope assay, and found dopamine at an approximate concentration of 300ng/g[25]. However, the enzymatic isotope assay is an indirect measure of dopamine, and may have inflated the actual number of detected molecules. Across all of these studies, the visual cortex had the lowest concentration of dopamine when compared to other cortical areas. For example, more frontal cortices typically had dopamine concentrations two to three times higher than the visual cortex ranging from 800-900ng/g as estimated by an enzyme isotope assay.

[a]**Receptor Radioautography**: Tissue is treated with a radioactive ligand of a receptor of interest. An image of receptor localization is produced by the decay emissions released from radioactive ligand.
[b]**Immunohistochemistry**: Specific antibodies generated against a target protein are used to localize protein in tissue sample via different strategies such as conjugated fluorophore or enzymatic stain.
[c]**Retrograde Tracer**: A chemical that is actively transported from terminal buttons to cell body, and is used to visualize neuronal connections.
[d]**High Performance Liquid Chromatography (HPLC)**: A liquid sample is passed through a column filled with solid phase material. Chemical components within the liquid sample are identified and quantified based on temporal interactions with solid phase.

In conjunction with evidence of dopamine and direct dopaminergic innervation, dopamine receptors have been detected in the visual cortex of the rat. Immunohistochemistry is the ideal methodology for localizing receptors at a cellular resolution. However, antibodies that target D1-like dopamine receptors often lack specificity, and an antibody that can reliably target D2-like receptors has yet to be developed. A lack of antibodies has hampered the anatomical localization of dopamine receptors across all species. Therefore, most studies that have localized dopamine receptors to cortical regions and specific cortical layers have relied on receptor autoradiography and radioactive ligands. Autoradiographic studies have shown that compared to cats, monkeys, and humans, rats have the fewest cortical dopamine receptors. Within the rat visual cortex, D1 receptors are most strongly represented in layers V and VI, and are approximately ten times more prominent than D2 receptors in most cortical layers. Though sparsely represented, D2 receptors are most strongly localized in superficial layers I and II[26–29].

Rats are not typically regarded as being visually oriented animals, and have relatively poor spatial acuity compared to higher mammals[30]. Therefore, rats may not be a suitable model to investigate the potentially subtle role that dopamine plays in visual processing. However, rats are inexpensive to maintain and can be easily subjected to surgical procedures that would be technically challenging to carry out in larger mammals. In an in vitro study on rat cortical slices, Saldate and Orrego (1977) found that slices taken from prefrontal areas release more irradiated dopamine when electrically stimulated than slices taken from rat occipital cortex[31]. Although this finding indicates the prefrontal cortex is equipped with more dopaminergic release sites, it also serves as a simple proof of principle that dopamine can be released from the neurons within the occipital cortex. A different study showed that the iontophoretic application of dopamine inhibited the activity of spontaneously firing neurons within rat visual cortex, but had varying effects on the responses of visually driven neurons[24]. Muller and Huston (2007) used microdialysis to show that visual stimulation increases the extracellular concentration of dopamine within the occipital cortex, but not in the temporal cortex. In addition, auditory stimulation did not affect the concentration of dopamine in the occipital cortex indicating that the increase in dopamine within the occipital cortex was visually mediated. Interestingly, cocaine administration augmented the amount of extracellular dopamine in both the occipital and temporal cortex[32]. Because cocaine administration can have pleasurable effects on rats, these findings might also suggest that the increase in occipital cortex dopamine was mediated by the hedonistic properties of cocaine. However, further experiments that test the effect of rewarding visual stimuli versus neutral stimuli on dopaminergic release in visual cortex still need to be conducted.

Evidence from Cat

Seminal studies on cat cortex helped confirm that dopamine afferents terminate in the mammalian cortex[33]. Later retrograde tracer studies confirmed that the cat visual cortex receives dopaminergic innervation from midbrain neurons. Following up on an initial finding that horseradish peroxidase (a first generation tract tracer) injected into the visual cortex resulted in VTA staining, Tork and Turner (1981) combined horse radish peroxidase and formaldehyde-induced catecholamine fluorescence to demonstrate that catecholaminergic (presumably dopaminergic) cells from the A10 nucleus of the VTA project to cat visual cortex[34]. Scheibner and Trk (1987) injected a more advanced retrograde tracer, wheat germ agglutinin conjugated with horseradish peroxidase (WGA-HR), into cat visual cortex, and quantified staining in five regions of the ventromedial mesencephalic tegmentum (VMT). The VMT is comprised of three other dopaminergic nuclei: the rostral linear (LR), interfascicular (IF), and central linear (LC) nuclei. In cat, the VTA contains two distinguishable sub-nuclei, which include the paranigral (PN) and the parabrachial pigmented (PBP) nuclei. After the cat visual cortex was injected with WGA-HR, substantial staining was found in the LC, LR, and PBP nuclei. Staining in the visual cortex was comparable to the motor and auditory cortices, but the prefrontal cortex exhibited almost eight times as much stain as the visual cortex[35]. While these tracer studies do not demonstrate dopaminergic release, they are strong evidence that the neurons within the VTA/VTM provide the cat visual cortex with dopamine. Although tracer studies in cats have provided evidence of the midbrain dopaminergic innervation of visual cortex, they did not provide any insight into which

layers of visual cortex are innervated by the midbrain. According to these reports, the tracers could be detected in all six layers of cortex after injection, which makes it unclear as to where the midbrain neurons actually terminate. Studies still need to be carried out that identify the laminar pattern of dopaminergic innervation in cat visual cortex.

Parkinson (1989) carried out an extensive investigation of dopamine in cat visual cortex. First, he compared the concentration of visual cortex dopamine between kitten (age 32-38 days) and adult cats using HPLC analysis. The visual cortex of adult cats contained approximately 93ng/g of dopamine, and the visual cortex of kittens was found to have approximately 30ng/g. This difference suggests dopamine may play a developmental role in the cat visual cortex. Next, he treated kittens with an intracerebroventricular injection of DSP-4, a neurotoxin that specifically targets noradrenergic neurons, and found that that DSP-4 treatment reduced the amount of norepinephrine in cat visual cortex by 80%. Interestingly, DSP-4 treatment did not significantly affect the amount of dopamine visual cortex[36]. A different experiment investigated the effect of monocular deprivation on visual cortex dopamine using HPLC, and found that adult cats had an average dopamine concentration of approximately 113ng/g[37]. Reader, Masse, and De Champlain (1979) used a radiometric assay to measure dopamine in different layers of the cat occipital cortex. Dopamine comprised approximately 40% of the catecholaminergic content. Norepinephrine constituted the other 60% of the measured catecholamines[38]. Using a similar radioenzymatic assay, Reader and Quesney (1986) found a similar ratio between dopamine and norepinephrine in cat occipital cortex, and found dopamine at an approximate concentration of 977ng/g[39]. Again, the discrepancy between the HPLC and radioenzymatic results are likely attributed to the detection method used to quantify dopamine. Overall, these results indicate that the concentration of dopamine in cat visual cortex is two to three times greater than the concentration of dopamine in rat visual cortex.

Expecting that dopamine was a functional neurotransmitter in cat visual cortex, Parkinson (1989) then sought to identify dopamine receptors in cat visual cortex via receptor autoradiography. He found that D1 receptors are present in layers I, II, III and VI, but failed to detect any D2 receptors. D1 receptors were more than two times more represented in layer VI than the other layers combined. In order to demonstrate that the detected D1 receptors were functional, Parkinson treated cellular homogenates of cat visual cortex with dopamine, and found that dopamine stimulates the production of cAMP in a dose dependent fashion. In a series of pharmacological experiments, Parkinson demonstrated that the exogenous dopamine was acting through D1 receptors rather through β-adrenergic receptors[36]. A separate autoradiographic study also found that D1 receptors were more strongly localized in the deeper layers of cat visual cortex, and failed to detect D2 receptors[13]. To date, no studies have successfully identified D2 receptors in cat visual cortex. The cat may not express D2 receptors in the visual cortex, or the radioactive ligands used in other species to localize D2 receptors fail to bind to cat D2 receptors due to species differences in receptor composition. Taken together, it appears as though both rats and cats express D1 receptors predominantly in the deep layers of visual cortex.

Cats utilize vision to effectively hunt prey and navigate their environment, and thus have evolved a sophisticated visual system. Therefore, cats offer researchers a more visually oriented animal for the study of cortical dopamine. However, the cat visual system has evolved to function optimally in low light conditions, and thus the architecture of cat visual cortex is intrinsically divergent from the primate visual cortex. Furthermore, cats are difficult to train via reinforcement, and this resistance to behavioral training has often restricted experiments that examine neural responses to anesthetized animals. Reader et al. (1976) found that that visual stimulation reduces the spontaneous release of dopamine from visual cortex neurons by 65% in anesthetized cats. In the same study, direct stimulation of the optic nerve also elicited a down regulation of spontaneous dopamine release in visual cortex. Following optic nerve stimulation, dopamine release from visual cortex neurons returned to basal levels[40]. In a follow-up study, Reader (1978) delivered dopamine to the visual cortex of anesthetized cats, and simultaneously recorded neural responses. Of the 35 neurons recorded, exogenous dopamine inhibited 22 of the neurons, and

had no effect on the other 13. The neurons that were unresponsive to exogenous dopamine were more restricted to the superficial layers of visual cortex, and the neurons that were inhibited by the exogenous dopamine were located in the deeper layers[41]. The responsiveness of visual cortex neurons to exogenous dopamine is in line with the dense population of D1 receptors in layer VI. Another study showed that electrical stimulation of the cat visual cortex can induce SNpc neurons to release irradiated dopamine, which further indicates that the visual cortex is functionally connected to midbrain dopaminergic structures[42]. These studies provide evidence that the cat visual cortex utilizes dopamine when processing visual information, and that dopamine release in visual cortex is correlated with the presentation of visual stimuli.

Evidence from Monkey

The dopaminergic afferents that innervate the visual cortex of monkeys are limited to layer I, and are rarely found in deep layers V and VI[43]. Rats exhibit the opposite pattern of laminar innervation, where most of the dopaminergic innervation is located in deep layers V and VI. Berger et al. (1988) used autoradiography to show that dopaminergic innervation is almost entirely restricted to layer I of visual cortex[44]. An immunohistological study on cynomolgus monkeys (Macaca fascicularis) and squirrel monkeys (Saimiri sciureus) observed tyrosine hydroxylase-immunoreactive fibers in layer I of primary visual cortex in both species. Tyrosine hydroxylase[e] is an enzyme in the synthetic pathway of dopamine. Importantly, tyrosine hydroxylase also marks noradrenergic neurons because dopamine is the precursor of norepinephrine. Layer 1 of the visual association area (area 18) contained slightly more catecholaminergic fibers than primary visual cortex (area 17)[45]. Using immunohistochemistry, Lewis et al. (2001) evaluated the distribution of the dopamine transporter in the cortex of male cynomolgus (Macaca fascicularis) monkeys. The dopamine transporter was sparsely expressed in the visual cortex, but was most prominent in layer I[46]. The dopamine transporter is the principle mechanism through which dopamine is cleared from the synaptic space, and is typically expressed on pre-synaptic dopaminergic terminals. Moreover, the dopamine transporter is rarely expressed on noradrenergic varicosities marked by the norepinephrine transporter[47]. Hence, the presence of the dopamine transporter in layer I suggests that the dopaminergic projections found in the monkey visual cortex comes from midbrain dopaminergic nuclei rather than brainstem noradgenergic nuclei. Although the presence of the dopamine transporter suggests midbrain innervation, tracer studies between midbrain neurons and visual cortex would be a more direct demonstration of midbrain innervation; these have yet to be carried out.

Like rodents and cats, there seems to be only sparse amounts of detectable dopamine in the visual cortex of non-human primates. In the mid-1970's, two separate studies utilized spectrophotofluorimetric analysis to measure dopamine in the visual cortex. Dopamine was present in the primate visual cortex at a concentration near or below 10ng/g[48,49]. Bjorklund et al. (1978) evaluated the concentration of dopamine within 22 cortical areas of the African green monkey (Cercopethecus aetiops) using radioenzymatic determination. Brain homogenate of the primary visual cortex contained dopamine at a concentration of 80ng/g. Dopamine levels were found to be many fold higher in the prefrontal cortices, ranging from 230ng/g to 450ng/g[50]. These results cumulatively indicate that the concentration of cortical dopamine is highest in the prefrontal and temporal neocortex, and seems to decrease along the fronto-occipital axis with only trace amounts of dopamine detectable in the visual cortex. As previously discussed, radioenzymatic assays tend to inflate the concentration of dopamine, so 80ng/g may be an overestimation. Hence, the monkey visual cortex seems to exhibit dopamine concentrations more similar to rat visual cortex than cat visual cortex.

Regardless of limited dopaminergic innervation and relatively low concentrations of dopamine, dopamine receptors are distributed across all six layers of the primate visual cortex. Lidow et al. (1991) used receptor autoradiography to assess the distribution of dopamine receptors in the visual cortex of the rhesus monkey. D1 receptors were detected in a trilaminar pattern with highest

[e]**Tyrosine Hydroxylase (TH)**: An enzyme that converts tyrosine into the precursor of dopamine, L-DOPA. Dopamine is synthesized from L-DOPA via aromatic l-amino acid decarboxylase (AADC). Dopamine is then converted into norepinephrine by dopamine beta hydroxylase (DBH).

densities in superficial layers I and II, middle sub-layer IVa, and deep layers V and VI. Other cortical areas such as the prefrontal and parietal cortex in addition to visual association area exhibited a bilaminar distribution of D1 receptors, which suggests organizational differences in dopaminergic innervation across cortical areas[51]. Another autoradiographic study in addition to a well-controlled immunohistological study have reported a similar distribution of D1 receptors in monkey visual cortex[13,52].

Lidow et al. (1989) showed that D2 receptors were located in monkey occipital cortex using 3H-raclopride as a radioactive ligand. D2 receptors were approximately two-times more prominent in frontal cortices than the occipital cortex[29]. Lidow et al. (1991) detected few D2 receptors in macaque visual cortex, and most of them were restricted to layer V. Camps, Kelly, and Palacios (1990) failed to detect any D2 receptors using 3H-CV205-502 as a radioactive D2 ligand. In the same study, 3H-CV205-502 successfully detected D2 receptors in the occipital cortex of humans and within other monkey brain regions, which suggests that the D2 receptors in the visual cortex of monkey may be below the detectable limit of 3H-CV205-502[13]. From these studies, it appears as if rats and primates have different distributions of D2 receptors in the visual cortex. Whereas D2 receptors are predominantly located in the superficial layers of rat visual cortex, D2 receptors seem to be restricted to the deeper layers in monkey visual cortex. This difference in receptor distribution may parallel a species difference between rats and primates in the laminar input to visual cortex. Even in layer V of monkey visual cortex, where D2 receptors are most represented, the density of D1 receptors is approximately 10 times greater than the density of D2 receptors[51]. A similar ratio of D1 and D2 receptors exists in cats and rats, which provides evidence that D1 receptors are the most abundant dopamine receptors in the mammalian visual cortex.

Monkey visual systems, particularly those of macaques, provide excellent models by which the cortical processing of visual information can be studied. First, the monkey and human visual systems are highly phylogenetically conserved. Second, monkeys can be trained to carry out complex visual tasks. Third, existing methodologies measure cortical neurotransmitter content and neural responses from the monkey visual cortex in awake, behaving animals. Finally, the response properties of macaque V1 neurons have been extensively studied, and therefore the modulatory influence of dopamine on V1 neurons can be easily tracked.

Despite the monkey being a premier model organism for visual processing, there have been few studies that have functionally tested the influence of dopamine on cortical circuits. A functional magnetic resonance imaging (fMRI)[f] study in macaque found that systemic application of L-DOPA, a dopamine precursor, increases energy metabolism in V1 disproportionally to the increase in cerebral blood flow, which results in a decreased BOLD response in that area. Interestingly, the same study showed that direct application of dopamine in V1 has no effect on neural activity, yet the distance of the recording electrode from the sight of dopamine application may explain the unresponsiveness of V1 neurons to dopamine in this study[53]. Another fMRI study found that a cue previously associated with reward, independent of visual stimuli, could down regulate monkey visual cortex activity. Pharmacological manipulation found that this inhibition was at least partially mediated by dopaminergic signaling[54]. Parkinson's disease is neurodegenerative disease in which dopaminergic neurons selectively die resulting in the severe motor deficits, cognitive decline, and eventually death. Motor deficits result from degeneration of midbrain dopaminergic neurons, but dopamine neurons in the retina also degenerate. In order to replicate Parkinson's disease in monkeys, midbrain dopamine neurons can be pharmacologically destroyed. Destruction of midbrain dopamine neurons in monkey caused deficits in vision independent of retinal destruction[55.]

[f]**Functional Magnetic Resonance Imaging (fMRI)**: A brain imaging techniques that detects changes in oxygenated blood flow.f

Evidence from Humans

Dopamine is converted into norepinephrine by the enzyme dopamine beta hydroxylase (DBH). By immuno-staining for both tyrosine hydroxylase and dopamine beta hydroxylase, dopaminergic neurons can be distinguished from noradgenergic neurons by the presence of tyrosine hydroxylase and the absence of DBH (i.e. TH+/DBH-). Gaspar et al. (1989) identified TH+/DBH- projections in the occipital lobe of six post mortem human brains, and found that most of them were located within layer I. The distribution of dopamine transporter-labeled fibers parallel TH+/DBH- projections, and are restricted to the superficial layers of human visual cortex[56]. The presence of the dopamine transporter in the superficial layers of visual cortex suggests that TH+/DBH- projections in the occipital lobe originate from midbrain dopaminergic nuclei. A neural track between human visual cortex and midbrain dopaminergic nuclei has not been explicitly exhibited. Unfortunately, retrograde tracers require active cellular transport, and cannot be utilized on post-mortem brain tissue. Therefore, a single neuron that tested positive for TH/the dopamine transporter and negative for DBH within visual cortex would present strong evidence in favor of midbrain innervation.

Experiments have been conducted that measure the concentration of dopamine or the distribution of dopaminergic markers in human visual cortex, yet experiments of this kind are rare due to a lack of readily available sample. Human brains collected under controlled conditions for neurochemical analysis are difficult to acquire. The most important condition is that brain must be extracted and frozen as soon as possible after death to minimize dopamine degradation. Studies have shown that as much as 50% of dopamine can break down within four hours of death[57–59]. The second most important condition is the brain is harvested from an individual void of any previous neuropathological disease. Javoy-Agid et al. (1989) evaluated the levels of dopamine in the cortex of two post-mortem human brains using a radioenzymatic assay, and found that the occipital cortex and the prefrontal cortex contained comparable concentrations of dopamine (~4.2ng/g and ~3.6ng/g, respectively)[60]. The ratio between prefrontal and occipital dopamine is unusual because the concentrations of prefrontal dopamine generally exceed the concentrations found in the occipital cortex. HPLC analysis of homogenates derived from human visual cortex found dopamine at a concentration between 8-9ng/g[43]. Two other studies utilized enzymatic approximations of dopamine, and measured dopamine at concentrations between 4x10-5-7ng/g[61]. The large differences between the amount of dopamine is likely due to differences in sample handling, the methodology used to measure dopamine, and uncontrolled differences between subjects such as age and cause of death. Though the results of these experiments vary, dopamine is clearly present in the human occipital lobe, and is present at a concentration comparable to monkey visual cortex.

Like the other mammals discussed, receptor autoradiography has been the most commonly used methodology to evaluate dopamine receptor distribution in human visual cortex. D1 and D2 receptors distribute throughout all six layers of human visual cortex[62]. D1 receptors exhibit a bilaminar pattern, and are most represented in superficial layers (I and II) and the deeper layers (V and VI)[63]. As found in rats, cats, and monkeys, D1 receptors are approximately ten times more common than D2 receptors[64]. Although sparsely distributed throughout visual cortex, D2 receptors seem to populate the superficial layers of cortex more densely than deeper layers[65]. This pattern is more reminiscent of the distribution of D2 receptors seen in rat rather than monkey, but studies on the cortical distribution of D2 receptor in the visual cortex of non-human primate are lacking. After comparing the distribution of dopamine receptors in the visual cortex to the rest of the cortical mantle, the human visual cortex seems to have relatively more dopamine receptors in visual cortex than other mammals.

The ultimate goal of studying dopamine in the visual cortex is to understand how dopamine influences the processing of visual information in the human brain. However, systematically testing the role of dopamine in the visual cortex requires invasive recording devises,

pharmacological manipulation, and even genetic alteration. None of these techniques can be readily applied to humans, and therefore causal research is restricted to model organisms. Transcranial magnetic stimulation, a noninvasive technique, could be utilized in humans to study the influence of dopamine on visual function, but these studies have not been carried out. These types of experiments will be valuable because research on how dopamine influences the processing of visual information in the human brain can be readily translated into practical applications. To date, the only evidence from humans that dopamine has a functional role in the processing of visual information comes from studies on people with Parkinson's disease. As mentioned previously, Parkinson's disease can affect retinal dopaminergic cells, and therefore studies that examine visual processing must control for possible retinal influences. In an attempt to control for retinal damage, experimenters will screen out patients with diminished visual acuity, and then employ other measures of visual function such as the ability to distinguish orientation, colors, and contrast[66–68]. From these studies, it is clear that dopamine signaling helps achieve optimal visual processing. However, none of these studies functionally attribute a lack of dopamine in visual cortex to any visual deficits.

Conclusions and Future Directions

When compared to other cortical regions, it is clear that the mammalian visual cortex expresses a very low amount of dopamine. What type of neural information could this small amount of dopamine signal to the visual cortex that would enable it achieve the optimal processing of visual stimuli? It has been proposed that midbrain dopamine neurons boost the processing of rewarding visual stimuli, and thus bias the visual system towards detection and identification in order to optimize the production of a behavioral output[69–71]. In agreement with this reward model, VTA stimulation and resulting dopamine signaling has been shown to specifically enhance the representation of rewarded frequencies within rat auditory cortex. More specifically, dopamine signaling was found to extend the area of auditory cortex responsive to a rewarded tone. Amazingly, areas of auditory cortex responsive to dissimilar frequencies were reduced in size[72]. It seems possible that signals from the VTA could serve a similar function in the visual cortex.

The reward model of cortical dopamine has been recently reconfigured to portray dopamine as a "reward prediction-error" signal. According to the reward prediction-error model, phasic dopamine release signals discrepancies between expected reward outcomes and observed reward outcomes[73]. Thus, future investigations of visual cortex dopamine should be designed to examine the effect of reward expectation and reward outcome on dopamine-mediated neural responses. If the prediction-error model were to hold true, dopamine signals would have to be delivered to the visual cortex on the millisecond time-scale, which is how quickly the visual system can encode visual information. This is because dopamine would need to report discrepancies in the hedonistic value of visual stimuli quickly enough to modulate the encoding of visual information.

Table 1 summarizes the anatomical findings reviewed in this paper. Limited dopaminergic innervation accompanied by wide spread dopamine receptor distribution is a common theme throughout each of the species discussed in this review. For example, the monkey visual cortex receives almost one hundred percent of its dopaminergic input in layer I, yet dopamine receptors are present in every layer of cortex. If this anatomical organization is accurate, than dopamine would be released from layer 1, and would have passively diffuse throughout the rest of the cortex to reach dopaminergic receptors. This is unlikely, as dopamine would have to traverse approximately 2.5mm of tissue. Although a chemical signal can functionally diffuse more than 1mm through brain tissue, a distance of .55mm is unlikely in the presence of active cleanup mechanisms such as the dopamine and norepinephrine transporter[74–76]. The norepinephrine transporter is found throughout visual cortex, and has a greater affinity for dopamine than the dopamine transporter[77]. Moreover, it could take seconds for dopamine to disperse throughout visual cortex, and this speed is not in coherence with the millisecond time-scale required by the reward-prediction error model.

Due to the peculiar arrangement of dopaminergic innervation and receptors, some researchers have proposed that there is an alternative source of dopamine in the mammalian visual cortex[78–80]. The locus coeruleus (LC) innervates all 6 layers of the visual cortex, and is the principle source of norepinephrine in the brain[81]. Dopamine is a precursor in the synthetic pathway of norepinephrine, and is thus present in noradrenergic neurons[82]. Therefore, it is possible that LC neurons can co-release dopamine and norepinephrine, and that these catecholaminergic neurons could activate dopamine receptors throughout all six layers of visual cortex. Although there is pharmacological evidence of dopamine and norepinephrine co-release, the existence of LC neurons that could release a functional dopaminergic signal has not been anatomically confirmed. Evidence of DBH+ neurons that also express the D2 auto-receptor would be strong evidence in favor of LC neurons releasing a functional dopaminergic signal.

Table 1: Dopaminergic System within Mammalian Visual Cortex.

Rat	
Laminar Innervation	V, VI
Dopamine Concentration	~6-14, 300
D1 Receptor Distribution	V, VI
D2 Receptor Distribution	I, II
Cat	
Laminar Innervation	unknown, from VTA/VTM
Dopamine Concentration	~30-93, 900
D1 Receptor Distribution	I, II, III, VI*
D2 Receptor Distribution	undetected

Monkey	
Laminar Innervation	I*, V, VI
Dopamine Concentration	~10-80
D1 Receptor Distribution	I, II, middle sub-layer IVa, V, VI
D2 Receptor Distribution	V
Human	
Laminar Innervation	I*
Dopamine Concentration	.00004-7
D1 Receptor Distribution	I, II, III, IV, V, VI
D2 Receptor Distribution	I*, II*, III, IV, V, VI

~Roman numerals indicate the layers of visual cortex. An * denotes that innervation or receptor distribution is most dense in indicated layer. Dopamine concentrations are presented as approximate concentrations in ng/g of protein analyzed

Although clearly mapping the dopaminergic innervation within visual cortex is critical, uncovering dopamine's influence on neural responses during visual processing is the ultimate goal. An ideal experiment would involve an animal carrying out a visual task and the experimenter simultaneously measuring dopaminergic release and neural responses at behaviorally relevant time-scales. In this way, different visual functions could be tested in conjunction with dopamine-mediated neural dynamics. The basic design of such of an experiment could be modified in order to demonstrate the influence of specific receptors, dopaminergic nuclei, and cortical layers.

Innovative new probes that combine fast scanning cyclic voltammetry (FSCV)[g] and traditional electrophysiology into a single device have been developed. These probes have been previously used to measure acetylcholine release and neural response within the primary visual cortex of awake, behaving rhesus macaques[83]. A variation of these probes could be used to record how dopamine influences cortical circuits. One potential obstacle is that the reduction potential, a measure of the tendency of a chemical species to acquire electrons, of norepinephrine and dopamine are so similar that the two molecules cannot be distinguished by conventional cyclic voltammetry. Moreover, the extracellular levels of norepinephrine are so many folds higher than dopamine in the visual cortex that the signal detected by cyclic voltammetry would be attributed entirely to the presence of norepinephrine. Until a technical advancement enables dopamine to be measured on the millisecond time-scale achievable only by FSCV, researchers will be restricted to separately measuring neural activity and the extracellular levels of dopamine.

Concluding Remark: There is much work to be done before solving the mystery of dopamine in the mammalian visual cortex. The known anatomy of the visual cortex dopamine is lacking, and additional studies that more clearly map out the dopaminergic innervation of mammalian visual cortex are in order. Moreover, preliminary data must be collected on dopamine-mediated neural responses within visual cortex. Advances in techniques designed to detect dopamine receptors and other dopaminergic markers will assist in the re-evaluation of the known dopaminergic input into visual cortex. Similarly, technical innovations in the electrochemical detection of dopamine will improve the temporal resolution of dopamine detection. Understanding the role of dopamine in the visual cortex could provide more than just insight into visual function. It will also shed light on the way dopamine influences all cortical circuits.

[g]**Fast scanning cyclic voltammetry (FSCV):** carbon-fiber microelectrodes are used to measure the oxidation and reduction of a target electrochemically active biogenic amines

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Glutamatergic Signaling in the Extended Amygdala: Implications for Psychiatric Disease

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Abstract

The regulation of glutamate in the brain is essential for maintenance of proper neurotransmission. Recently, a growing body of evidence has implicated dysregulation of the glutamatergic signaling system as a precipitating factor for several neuropsychiatric disorders, including depression, generalized anxiety, and addiction. While a number of higher order brain structures have been studied for how changes in their glutamatergic signaling can alter cognitive functions and emotional processing, relatively less attention has been paid to the role of glutamate in brain regions that serve as relay centers for reward and stress-based neurocircuitry. The extended amygdala is a key integrator of stressful and rewarding stimuli, and depends on proper glutamate signaling to participate in the control of behavioral and functional responses to such information. Here, I will review the current literature regarding the role of glutamate in the extended amygdala, and how changes in its signal in response to acute and chronic negative stimuli may be involved in the pathology of neuropsychiatric disease.

Introduction

Glutamate is the primary excitatory signaling molecule in the brain, and is essential for the maintenance of a number of structural and functional synaptic processes important for learning, memory and general cognitive function. Changes in glutamate signaling, particularly in response to stress, have long been suspected to be detrimental to mental health[1,2,3]. A number of the symptoms of psychiatric illnesses that affect cognition and emotional processing have been linked to the effects of unregulated excitatory signaling, including the modulation of responses to stress-inducing and motivated behaviors that are classically seen to go awry in depressive, anxiety and addiction-related disorders[1,2,3,4]. This suggests that glutamate regulation is important in areas involved in the reward-seeking and stress-response neurocircuitry. The brain regions that compose the "extended amygdala", a macrostructure of the limbic system, are anatomically positioned to serve as regulators of both of these networks[5,6,7,8]. Additionally, a growing body of work has shown that glutamate appears to play a pivotal role in regulating intra- and intercellular communication in the extended amygdala, as well as in a number of the brain regions it projects to that are implicated in processing and responding to stressful and rewarding stimuli[9,10,11,12]. A better understanding of how glutamate is able to modulate signaling within the extended amygdala may therefore provide insight into the underlying physiological changes that occur in psychiatric illness, specifically in response to outside factors such as prolonged stress. This review will discuss the current understanding of glutamatergic signaling alterations in disease states, as well as findings regarding the role of glutamate in the circuitry of the extended amygdala. We additionally address how changes to this signaling may be precipitated and its implications for neuropsychiatric illness.

The Glutamatergic Signaling System and Stress-induced Disease

Glutamate exerts its activity at a number of different receptors located throughout the nervous system, primarily consisting of two main classes: the metabotropic glutamate receptors (mGluRs) and the ionotropic glutamate receptors (iGluRs)[13]. The mGluR family is composed of roughly eight different types (mGluRs1-8) collected into three distinct groups (I, II or III), based on their associated second messenger and ligand sensitivity[14]. The group I mGluRs are known to increase excitatory neurotransmission in a Gq G-protein coupled receptor (GPCR) dependent manner, and are primarily located postsynaptically[14]. The groups II and III mGluRs, by contrast, are inhibitory (via Gi dependent GPCR signaling) and tend to be located presynaptically[14].

Likewise, the iGluRs can be divided into four distinct classes based on physiology and ligand responsiveness, and include the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and the delta receptors[13]. The AMPA receptors (AMPARs) serve as the primary charge carriers of excitatory neurotransmission, allowing for initial depolarization of the cellular membrane necessary to bring the cell to threshold for the firing of an action potential[13]. The NMDA receptors (NMDARs) require an initial membrane depolarization in order to respond to further changes in excitatory tone at the synapse, but are highly sensitive to these changes, allowing them to readily modify excitatory signal strength[13]. Activity dependent glutamatergic signaling through both mGluRs and iGluRs has long been known to be a key mediator of synaptogenesis and synaptic plasticity. Specific forms of plasticity, such as long term potentiation (LTP) and long term depression (LTD), can be modulated by either major class of glutamate receptor, and ultimately result in either increased or decreased activation of the NMDARs and subsequent insertion or removal of AMPARs at synapses to promote stronger or weaker excitatory signaling[13,15,16]. The precise regulation of synaptic structure and function via these means has been shown to be critical for mediating cognition, learning and memory, and behavioral outputs[1,13].

Considering how essential these synaptic and signaling processes are to general neurological function, it is not surprising that disruption of proper glutamatergic signaling can lead to severe deficits and chronic neuropsychiatric disease states. Schizophrenia (SCZ) is one of the most prominent disorders that has been suggested to have a basis in dysregulated excitatory signaling, with numerous studies pointing to a change in NMDAR function and increased glutamatergic tone as possible underlying causes of its cognitive and emotional deficits[17,18]. Genetic alterations to the glutamate system have been shown across clinical and animal based studies to be precipitating factors for changes in excitatory signaling and mental disease, as have a number of extrinsic factors, the most prominent of these being stress[19,20]. Stress has been shown to disrupt glutamatergic signaling in brain regions associated with cognition and emotional processing, such as the prefrontal cortex (PFC) and hippocampus (HIPP), producing deleterious effects on the structure and function of excitatory synapses[21,22]. Multiple studies have shown a connection between decreases in excitatory signaling in regions central to the regulation of emotional valance and cognition and reported increases in psychiatric symptoms (e.g. depressed mood, altered motivational drive and lack of emotional control) as well as altered animal performance in behavioral tasks with face validity for affective disorders such as depression, generalized anxiety and addiction[2]. By contrast, brain regions that participate in the regulation of stressful stimuli and motivated behaviors have been reported to show enhancements in excitatory activation, as well as increases in synaptic structure/function, in disease states precipitated by extrinsic factors like stress[23,24]. Examination of the amygdala in stress-associated disease states has shown hypertrophy of the region and increased dendritic length and spine density in its basolateral nucleus (BLA), while electrophysiological recordings from the region following chronic stress show increased spontaneous excitatory signaling[25,26,27]. Recent work has also shown that direct optogenetic activation or inhibition of glutamatergic synapses within the amygdala can enhance or reduce the retention of depressive or stress-associated phenotypes, respectively[28,29]. Bidirectional changes in the strength of the excitatory circuitry that mediate responses to positive and negative stimuli thus appear to underlie many symptoms of these affective disorders, suggesting that restoration of the basal excitatory state within these networks could alleviate behavioral and functional changes associated with disease. A better appreciation of the glutamatergic circuity of limbic brain regions that are associated with circuitry and its regulation could thus provide greater understanding of the role for glutamate in disorders such as depression, anxiety and addiction.

Anatomy of the Extended Amygdala and its Glutamatergic Circuitry

While higher brain regions and those more classically associated with the control of emotions and motivated states have been more extensively characterized for changes in their glutamatergic signaling in disease states, regions such as the extended amygdala are just

starting to be explored in more detail[9,30,31]. The extended amygdala serves as a key integrator of stressful and rewarding stimuli within the brain, and is comprised of three core structures: the bed nucleus of the stria terminalis (BNST), the central nucleus of the amygdala (CeA) and the shell of the nucleus accumbens (NAc-Sh)[32,33] . These components are highly interconnected with one another, as well as with a number of other regions that participate in the responses to stress and motivated behaviors, such as the hypothalamic-pituitary-adrenal (HPA) axis and ventral tegmental area (VTA)[34,35,36]. Reciprocal connections exist between the BNST and CeA, with the CeA exerting inhibitory regulation over the BNST's activity[37,38,39]. Responses to stressful stimuli are thought to be mediated not only by these connections, but also through projections the BNST sends to the paraventricular nucleus (PVN) of the hypothalamus, where it can influence the release of stress hormones like cortisol[36,40]. Rewarding stimuli, by comparison, is thought to be regulated through the BNST's projections to the NAc-Sh, its excitatory and inhibitory projections to the VTA, and its aforementioned hypothalamic connections[8,34,35]. The extended amygdala is also densely populated by a number of cells that produce and respond to neuropeptides that have been extensively studied for their roles in regulating stress and reward behaviors, including corticotropin-releasing factor (CRF), neuropeptide Y (NPY) and several others[41,42,43]. CRF is prominently expressed in the BNST and CeA, and both regions send numerous CRF projections to one another, as well as to other limbic structures[44,45]. Signaling via CRF within the extended amygdala has also been linked to the regulation of a number of behavioral responses[46,47]. Together, the myriad connections and heterogeneous cells populations within the extended amygdala suggest its unique role in modulating behavioral responses to stressful and hedonistic stimuli.

The majority of neurons found within the components of the extended amygdala express the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), and thus send and receive many inhibitory connections[48,49]. However, the extended amygdala also possesses numerous excitatory afferent and effect projections. Glutamatergic inputs to the region come from multiple cortical and limbic regions, including the BLA, HIPP, PFC, amygdala, insular cortex and the limbic cortex, while its glutamatergic outputs principally project to the VTA, as well as several other regions[38,50,51]. Many of the regions sending and receiving glutamatergic projections to and from the extended amygdala have been identified as important centers of emotional and cognitive processing, and critical for regulating the behavioral responses to stressful and rewarding stimuli[2,3]. Further, it is suggested that changes in glutamatergic signaling within these regions may be related to the altered responses to such stimuli that are noted across a number of neuropsychiatric diseases[1,2,3].

Glutamate Signalin in the Extended Amygdala is altered in Disease States

Given its interconnectivity with numerous sites of emotional, cognitive and functional processing, and its participation in regulating the circuitry of stress, anxiety and drug-seeking behaviors, it is not surprising that altered excitatory activity within the extended amygdala has been suggested to occur in affective disorders. Indeed, direct inhibition of AMPAR-dependent glutamatergic signaling in the BNST (but not CeA) via injection of the channel blocker NBQX has been shown to decrease normal acquisition of fear conditioning in rodents, while injection into regions of the BLA that send excitatory projections to the BNST enhances fearful and anxiety-like responses[37]. The emergence of addictive tendencies has also been implied to depend on changes in the strength of glutamatergic projections innervating the VTA and HPA axis. Several lines of research have purported that the extended amygdala is an important source of the glutamatergic signal that mediates these behaviors[2,30]. Much interest has additionally been given to the modulatory effects of the neuropeptide signaling system on glutamatergic signaling following HPA axis activation and in response to neuroadaptive changes occurring in disease. The extended amygdala is heavily populated by neurons that either produce or express receptors for neuropeptides like CRF, NPY, enkephalin and more, and several studies have indicated these cell populations may exert a degree of bidirectional control over the activation or inhibition of the extended amygdala's stress and reward related

circuitry[41,45] . The following section will discuss the implications for altered glutamate signaling in the extended amygdala in precipitating or exacerbating the maladapative changes classically associated with affective and drug related neuropsychiatric disease. The interplay of neuropeptides and glutamatergic modulation in these regions will also be examined.

The Bed Nucleus of the Stria Terminalis

Due to its well established role as an integrator of affective and stressful stimuli and specific glutamatergic connections to multiple regions key to regulating behaviors associated with these stimuli, altered excitatory signaling in the BNST is of particular interest to the study of psychiatric disease[2,30]. The BNST has numerous glutamatergic connections to several brain regions involved in the regulation of fear, stress and reward related stimuli (PFC, HIPP, VTA, BLA and insula), suggesting that modulation of the glutamate signal both at these projections and within the BNST itself can impact behavioral responses to these stimuli[38,50,51]. Conrad et al. demonstrated that animals exposed to mild chronic stress and intermittent injection of the stress hormone cortisol displayed significant increases in anxiety-like behaviors and an associated reduction in BNST LTP[52]. The same group also found that BNST synaptic plasticity was significantly reduced in animals exposed to chronic stress and chronic ethanol exposure, suggesting that an increased anxiety-like response to stress may correlate with decreased intercellular BNST excitatory drive[53]. Kim et al. elegantly demonstrated the effects of glutamatergic regulation as well via a combination of electrophysiological and pharmacological studies that showed that both direct infusion of glutamate receptor specific antagonists into the dorsal BNST and optically invoked inhibition of BNST excitatory signaling were able to produce robust decreases in anxiety-like behaviors in the elevated plus maze and the open field test[54]. The involvement of the BNST in mediating the stress-induced and reinforcing behaviors associated with chronic ethanol drinking has also been repeatedly linked to similar changes in glutamatergic neurotransmission[55,56,57]. Indeed, acute application of ethanol to brain slices containing the BNST has been shown to reduce NMDAR-dependent EPSC amplitude and NMDAR-isolated field potentials[55]. Separate but related studies from Kash and Wills expanded upon these ethanol specific effects at NMDARs in the BNST, showing that expression of NMDARs containing the GluN2B subunit within the BNST increases following chronic ethanol drinking, and that regional ablation of GluN2B-containing NMDARs blocks both acute and chronic alcohol induced reductions and enhancements, respectively, in BNST LTP[10,57]. Changes in NMDAR- specific glutamate signaling in the BNST have also been linked to mediating other stress-related behaviors. As recently shown by Holleran et al., animals lacking BNST GluN2B display a reduced depression-like phenotype in the novelty induced hypophagia (NIH) task, effects which can be replicated in wild type animals following administration of the GluN2B specific antagonist, Ro25-6981[58]. Structural and neuroadaptive changes in glutamate receptor makeup and synaptic strength at the level of the BNST may thus play a role in regulating responses to prolonged stress or chronic drug use.

CRF within the BNST has also been linked to the modulation of its glutamate signaling[43,44,45,46,59]. Silberman and colleagues demonstrated that exogenous application of CRF enhances the spontaneous EPSC frequency of BNST neurons projecting into the VTA, and that application of the CRFR1 antagonist NBI27914 can block increases in such excitatory signaling[60]. Indeed, it was also discovered that CIE treatment in animals was also able to enhance excitatory drive onto VTA-projecting BNST neurons, occluding the effects of exogenous CRF application[60]. This increase in BNST-VTA glutamatergic signaling in response to CRF suggests that chronic stress may enhance the BNST's excitatory input to the VTA (BNST-VTA Glu+), potentially inducing maladaptive changes typical of drug dependency and withdrawal. Jennings, Kash and Stuber elegantly expanded on the idea of enhanced glutamatergic signaling at BNST-VTA Glu+ and its implication for altering stress and reward-seeking responses in a recent study using optogenetic stimulation of both the glutamatergic and GABAergic VTA projections. In vivo recording of both BNST-VTA Glu+ and BNST-VTA GABA+ cell populations showed that excitatory signaling of the glutamatergic cells specifically was

enhanced during stressful events, while GABAergic cell activity was suppressed[9]. Selective activation of the BNST-VTA GABA+ projections was also found to decrease avoidance behaviors real time in conditioned place preference assays, while activation of the glutmatergic projections produced adverse and anxiogenic behaviors reminiscent of the effects of CRF application[9]. Evidence such as this further supports the notion that modulation of the BNST's glutamatergic signal is correlated with the neurochemical and neuroadapative effects of stress-inducing stimuli and drugs of abuse. Chronic dysregulation of this glutamate signal suggests the exacerbation of these effects, and thus the precipitation of pathologies associated with affective disorders.

The Central Nucleus of the Amygdala

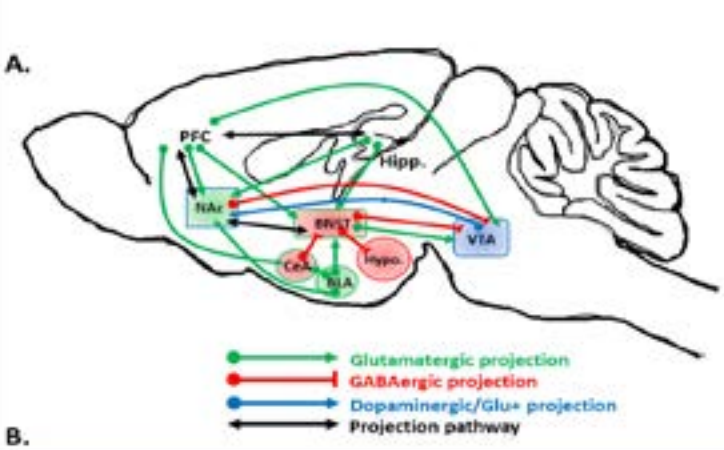
The CeA serves as the primary site for intra- and extra-amygdalar glutamatergic inputs, receiving afferents from the BLA as well as the thalamus, cortex and brainstem at its lateral subdivision[61,62]. Additionally, it is one of the main output nuclei of the amygdalar complex, and projects via its medial subdivision to multiple brain structures involved in fear, arousal, and stress responses, pointing to an important role for the CeA in the regulation of these behaviors[63]. A number of studies have shown that extracellular glutamate increases in the CeA in response to stress, resulting in enhanced anxiogenic behaviors and fear conditioning in rodent models[64]. Significant alterations in CeA glutamate signaling have also been correlated with increases in drug-seeking and drug associated reward behaviors, and has been extensively studied in animal models of binge drinking in alcoholism[65,66,67]. Rodents exposed to chronic intermittent ethanol (CIE) treatment show increased alcohol self-administration and overall motivation to drink, and the blockade of select glutamate receptors in the CeA has been shown to inhibit the reward seeking behaviors associated with increased drinking, indicating that chronic drinking increases glutamate in the CeA[67]. Roberto et al. demonstrated this physiologically, showing that both CIE and direct ethanol superfusion produced marked depression in CeA NMDA- and non-NMDA-dependent excitatory postsynaptic potentials (EPSPs), paired pulse facilitation (PPF), and overall excitatory drive in CeA neurons, suggesting an alcohol-induced increase in CeA glutamate release that may lead to decreased ionotropic glutamate signaling (via habituation or desensitization to increased glutamate tone) and a net depression of the neuronal activity in CeA cells[68].

Glutamatergic signaling within the CeA has also been shown to be heavily influenced by CRF, one of the principle stress neuropeptides that is heavily expressed within the extended amygdala[11,69]. The CeA in particular possesses a large population of CRF cells (many of which project to the BNST), as well as a large number of cells that are responsive to CRF via one of its major receptors, CRFR1[46]. Activation of these receptors has been shown to potentiate the glutamatergic connection between the CeA and the BLA[70]. Silberman et al. has also demonstrated that CRFR1 activation enhances excitability within the CeA using bath application of CRF and optically evoked stimulation of the glutamatergic projections to the CeA[71]. Superfusion of CRF was shown to produce marked increases in spontaneous EPSCs, while optically evoked activation of glutamatergic afferents to the CeA showed little change in basal excitatory signaling following CRF wash-on, suggesting a presynaptic mechanism for CRF enhanced glutamatergic signaling in the CeA[71]. Direct injection of CRF into the CeA has also been shown to produce increased extracellular glutamate levels, similarly to the changes in CeA glutamate concentration seen in response to both external stress and drug administration[72]. Further behavioral studies have also expanded upon CRF's influence on CeA glutamatergic signaling, showing that withdrawal from chronic drug exposure can enhance CRFR1 induced LTP in the CeA, and that the blockade of CeA CRFR1 can relieve the negative behaviors that are often symptomatic of withdrawal, possibly by preventing further glutamate release and bringing glutamatergic tone back to basal conditions[73,74]. These findings suggest that the glutamate signal within the CeA can be subject to multiple degrees of modulation, and is particularly sensitive to prolonged exposure to external stressors and/or drugs of abuse. Neuroadaptation of the excitatory circuitry of the CeA resulting from either dysregulation of stress hormone signaling or chronically altered glutamatergic tone may thus underlie the many of the changes in motivated behavior and stress/anxiety responses noticeable in addicted and neuropsychiatric patients.

The Nucleus Accumbens

Though it is often anatomically considered a separate entity from the extended amygdala, the NAc (particularly the NAc-Sh) has extensive functional connectivity with the extended amygdala, hypothalamus, and other limbic structures, and participates in regulating a number of behaviors and stimuli that overlap with them[3,75,76]. The NAc is an important regulator of the VTA (VTA-NAc Glu+ & dopamine), and integrates excitatory information from the PFC (PFC-NAc Glu+) and HIPP (HIPP-NAc Glu+), affording it express control over motivated and reward seeking behavior[12,75,76]. Multiple studies have emerged showing that stress is able to alter the glutamatergic signaling in the NAc, resulting in reductions in associated hedonistic and stress-related behaviors, particularly via altered excitatory activity at select populations of medium spiny neurons (MSNs) in response to dopaminergic cues[77,78]. Indeed, animals exposed to chronic stress have shown decreases in AMPAR mediated excitatory drive on those medium spiny neurons positive for the D1 dopamine receptor (D1-MSNs) in electrophysiological recordings, as well as associated decreases in reward-seeking behaviors[79]. Recently, Francis et al. elegantly expanded on this, further implicating the effects of external stress on glutamatergic signaling in the NAc. Chronic stress was shown to produce similar decreases in the frequency of D1-MSN excitatory postsynaptic currents (EPSCs) and overall decreases in intrinsic cell excitability in ex vivo and in vivo recordings[80]. Bidirectional control of excitatory drive onto the MSNs achieved via optical stimulation of either channelrhopopsin 2 (ChR2) or injection of the designer drug clozapine-N-oxide (CNO) to activate the inhibitory designer receptor exclusively activated by designer drug (DREADD) hM4(Gi), produced either increased or decreased EPSC frequency and amplitude, which were respectively able to diminish or exacerbate anxiety- and depressive-like behavior in the awake animals[80].

Glutamatergic signaling at projections from the PFC, HIPP and BLA to the NAc (BLA-NAc Glu+) are also noticeable altered in other disease states, such as addiction[81,82]. While dopamine is the key neurotransmitter known to modulate changes in addictive tendencies at the level of the NAc, a number of studies have proposed a prominent role for glutamatergic control over these behaviors as well[81,82]. These studies all point to a net increase in synaptic plasticity and excitatory signaling in the NAc following chronic drug use, resulting in a shift in the homeostatic balance of glutamate towards the opposite extreme from what is seen in the cases of anxiety and depressive-like disorders in regards to NAc glutamate. Both Stuber and Bonci have directly demonstrated the ability of enhanced glutamatergic signaling from these inputs to increase both excitatory activity in the NAc and reward-seeking behavior. Light evoked stimulation of BLA-NAc Glu+ and HIPP-NAc Glu+ inputs was shown to produce increases in AMPAR/NMDAR ratio and EPSC amplitude in the NAc, indicating increased insertion of AMPARs at the synapse and functional enhancement of the synapses, and corresponded to increases in optical self-stimulation attempts when animals were trained to directly activated these glutamatergic pathways, suggesting an increased response to motivational stimuli[12,83]. By comparison, stimulation of PFC-NAc Glu+ does not appear to drive general increases in motivated behavior in addiction models, but instead to regulate the relapse of drug-seeking behaviors that follow initial extinction or withdrawal[12,83]. Targeting of this connection in cocaine-addicted animals has validated these findings. Inhibition of PFC-NAc Glu+ was shown to prevent reinstatement of cocaine seeking in self administration paradigms as well as produce measured rises in the concentrations of glutamate at the NAc[84]. Collectively, current evidence from the field suggests a key role for glutamate at the NAc and its associated projection areas in the bidirectional control of behavioral responses to stressors and rewarding stimuli. The dysregulation of this control may thus be relevant to the disease state in several disorders that show a decreased ability to control affect and motivated response.



B.

DISORDER	BNST-VTA	BLA-BNST	BLA-CeA	VTA-NAc
Depression	↑	↑	↑	↓
Stress/ Anxiety	↑	↑	↑	↓
Addiction	↓	↑	↑	↑

Figure 1 *Key Glutamatergic Projections of the Extended Amygdala.* Top: Sagittal view of the mouse brain showing the major glutamatergic projections btw components of the extended amygdala and surrounding brain regions known to be key for the regulation and processing of rewarding and affective stimuli. (BNST= bed nucleus of the stria terminalis, Hypo= hypothalamus, BLA= basolateral nucleus of the amygdala, CeA= central nucleus of the amygdala, NAc= nucleus accumbens, VTA= ventral tegmental area, Hipp= hippocampus, PFC= prefrontal cortex). Bottom: table outlining the predicted changes in glutamatergic signaling at key excitatory pathways between these regions that are thought to be affected by an array of neuropsychiatric disorders (↑= enhanced signaling, ↓= decreased signaling).

Glutamate as a Potential Target for Treating Psychiatric Disorders

Evidence for the dysregulation of the glutamatergic system across a number of different psychiatric diseases has generated much interest in potential therapies aimed at restoring glutamate homeostasis[85]. Ongoing efforts within the field of schizophrenia have focused on the development of mGluR allosteric modulators and AMPAR antagonists with the goal of either enhancing the function of NMDARs expressed on GABAergic interneurons or the reduction of downstream excess glutamate release, respectively[86,87,88]. A tentative link has also been established between the use of memantine and other low affinity NMDAR antagonists and the reduction of physical withdrawal symptoms, suggesting that glutamate targeted interventions may hold some potential for assisting patients suffering from substance addiction[89,90,91]. The NMDARs have represented a particular area of interest for the treatment of depression due to the rapid and robust antidepressant-like effects produced both in patients and animal models of the disorder following the administration of NMDAR antagonists[58,92,93,94]. Ketamine has long served as a primary example of the efficacy for NMDAR modulation in the treatment of depressive-disorders. However, new evidence may suggest activity for ketamine in an NMDAR-independent fashion, implicating AMPARs as an alternative target for the drug's therapeutic effects[95]. Regardless, a number of studies utilizing both genetic and pharmacological inhibition of NMDARs have been shown to have positive implications for treating neuropsychiatric disease[85,89]. The NMDARs are heteromeric proteins that are composed of an obligate GluN1 subunit and a combination of either multiple GluN2 (A-D) or GluN3 subunits, and various subtypes are present throughout the brain, in particular at limbic structures where their postsynaptic expression patterns have been found to be quite high[96,97]. Targeting of subunit specific subtypes of NDMARs has received more attention as a therapeutic avenue for treating multiple psychiatric and general neurological disorders as of late, as exampled by several recent pharmacological and behavioral studies. Miller and Hall demonstrated that genetic deletion of the GluN2B subunit in the PFC of rodents is capable of producing antidepressant-like behaviors within the forced swim and tail suspension tasks, and that these effects are also produced via administration of Ro25-6981 to wild type animals[93]. Similarly, genetic knockout of GluN2A has been shown to produce a reduction in depressive behaviors[20]. Miyamoto and colleagues have also shown constitutive ablation of GluN2D, a less abundant subunit possessing several interesting physiological properties, appears to produce an antidepressant-like and anxiolytic

phenotype in adult mice in the forced swim, elevated plus maze and light/dark box tasks[98]. Interestingly, several studies have shown that altered GluN2B-NMDAR excitatory activity in the BNST correlates with the structural and functional changes often associated with chronic alcohol drinking and the regulation of depressive and anxiety-like behaviors, suggesting that altered NMDAR signaling in the BNST may be of particular interest in the study of certain disease states[10,57,58,99]. Collectively, these studies demonstrate that the glutamatergic system has proven to be a highly efficacious and optimal therapeutic target for the treatment of a number of psychiatric disorders. The continued development and study of newer and more specific compounds to glutamatergic receptors and receptor subtypes may also provide a greater understanding of the role of glutamate neurotransmission in the regulation multiple behaviors, and how this signaling system may be altered across different disease states.

Conclusion

The relationship between proper glutamatergic signaling and the regulation of functional neurotransmission and behavioral response is well established, and the implications for the dysregulation of this system have extended to multiple psychiatric conditions[2,4,18]. Converging lines of research indicate that particular attention should be paid to the maintenance of the glutamatergic signal within regions of the central nervous system that are anatomically and functionally situated at the crossroad of the primary neurocircuitry networks that appear most affected by these disorders. The extended amygdala has been postulated to be such a region. Mounting evidence supports this notion, showing that the plasticity of the extended amygdala's excitatory inputs and outputs can be hijacked by the maladaptive functional and structural changes imposed by mental disease[6,9,100]. For instance, the transient increases or decreases in excitatory signaling in the NAc following either rewarding or stressful events that appear to become chronically exacerbated in animal models of addiction and anxiety suggests a bidirectionality to glutamate homeostasis that is critical to preventing either extreme from winning out[81,101]. Likewise in the CeA, increases in both extracellular glutamate and the glutamatergic input to the region that enhances anxiogenic and reward-seeking behaviors can also become either hypo- or hyper-activated under chronic exposure to stimuli classically known to precipitating these disease states[29]. Glutamatergic signaling in the BNST also shows a similar regulation of affective stimuli, with enhanced glutamate correlating to increased stress responses and adversive behavior, and chronic states of stress and drug dependence producing altered sensitivity to glutamate and differential increases and decreases in intrinsic excitatory signaling that alter response to external stimuli[37,54,60]. These observations suggest that glutamatergic signaling may have opposing effects across the extended amygdala, allowing its multiple components to modulate one another as well as regions that are functionally connected to them within the HPA axis and greater limbic system. The high expression of GABA and vast inhibitory connections found at the levels of inter- and extra- extended amygdalar circuitry also indicate that glutamatergic signaling onto and within this region may be instrumental to modulating the inhibition or "dis-inhibition of inhibition" of specific GABAergic cues that are important for mediated divergent aspects of emotional and motivational processing[9,31,102,103].

A number of important questions still exist regarding how exactly the glutamatergic signal in the extended amygdala may modulate the emotional and motivational circuitry of the brain. Though many of the studies discussed here demonstrated that both precise control over the glutamatergic projections of the BNST, CeA and NAc, as well as broad alterations of glutamatergic tone and responsiveness at a receptor level either underlie or can replicate the pathological effects of addiction, depression or general anxiety, how these changes are achieved at the level of micro- and macrocircuitry has yet to be fully elucidated. The successful implication of opto- and chemogenetics and several other tracer based technologies within the study of the extended amygdala offers some insight into the future of these endeavors. The dissection of these inter- and extra-regional circuits can be achieved and utilized to better understand the influence of the extended amygdala on stress-induced and reward-seeking behaviors[9,12,83,103]. Likewise, further examination of the specific effects of the glutamatergic signal at GABAergic cell populations locally within the extended amygdala and at associated

areas connected to it will be important to develop a better understanding of how inhibition or disinhibition of particular parts of the stress- and reward-based circuitry can be influenced by glutamate, and how this translates to changes in behavior that can be potentially exacerbated by changes to this circuitry in disease states. Further study of the extended amygdala and the role of its glutamatergic signaling in regulating behaviors in both basal and diseased states is thus justly warranted, and may offer the promise of more specific and potentially efficacious targets for treating addiction, depression and anxiety-related neuropsychiatric disease.

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Additional Published Reviews

A few students in the 2016 qualifying class have had their qualifying exam reviews published in external journals. Here are the citations for those published reviews:

1. Golovin, R. M. & Broadie, K. Developmental experience-dependent plasticity in the first synapse of the Drosophila olfactory circuit. J. Neurophysiol. 116, 2730–2738 (2016).

2. Maksymetz, J., Moran, S. P. & Conn, P. J. Targeting metabotropic glutamate receptors for novel treatments of schizophrenia. Mol. Brain 10, (2017).

3. Shafer, R. L., Newell, K. M., Lewis, M. H. & Bodfish, J. W. A Cohesive Framework for Motor Stereotypy in Typical and Atypical Development: The Role of Sensorimotor Integration. Front. Integr. Neurosci. 11, (2017).