Vanderbilt Reviews Neuroscience

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LETTER FROM THE EDITORS

Dear Friends and Colleagues of the Vanderbilt Neuroscience Community,

It is with great enthusiasm that I present to you the 10th Volume of Vanderbilt Reviews Neuroscience (VRN), a journal showcasing the work of the newly-minted class of Ph.D. candidates in the Vanderbilt Neuroscience Graduate Program. Over the past decade of its existence, the VRN has evolved to reflect the changing needs and wants of our neuroscience community while still preserving many foundational traditions. Importantly, at its core the VRN remains trainee-centric, with the contributions and content coming predominantly from current graduate students. This year marks the first that, in place of volunteers, Associate Editors were elected to their position through the Neuroscience Student Organization (NSO) to provide support to the Editor-in-Chief. One of the recent changes to the VRN relates to the publishing process: we are now using electronic media as our primary mode of dissemination.

In Volume 10, you will find reviews from the talented qualifying class of 2017 – comprising students from the IGP, the direct admit program, MSTP students, and one of our first joint J.D./Ph.D. candidates. Topics include the role of parvalbumin interneurons in addiction, injury, and disease states; understanding how sensory systems work together to encode information and utilizing multisensory processing concepts to create novel rehabilitative tools; the contribution of various protein complexes, cotransporters, and receptors to epilepsy; and how neuroscience can inform the assumptions behind existing evidentiary rules used in the courtroom.

As always, the students in our program are highly productive publishers. In this volume we highlight a number of these first-author manuscripts published during the 2017-2018 year.

As a community our efforts extend far beyond the lab. Our Vanderbilt Brain Institute (VBI) Interim Director, VBI Director of Graduate Studies, NSO President, and NSO Community Outreach Coordinators, were kind enough to provide messages with updates on just some of these efforts.

There are several individuals to whom I would like to extend my sincere gratitude. Francis Cambronero and Randy Golovin, our first elected Associate Editors, have poured a tremendous amount of work into editing several reviews and coordinating revisions with busy graduate students – all while balancing their own lab schedules; I am grateful to them for their assistance in helping me get the reviews into the condition you will see here. I appreciate the advice from our previous Editor-in-Chief, Robin Schafer, who openly shared her experiences of what did and did not work in producing this publication. I would like to acknowledge Tin Nguyen for providing the cover art for this issue. I am indebted to Beth Sims, who has been nothing short of instrumental in the creation of this issue. Beth, among many other tasks, spearheaded the collecting of reviews from students and the formatting of the entire issue, which is by no means a small feat. Of course, this publication would not be possible without the hard work and the timely cooperation of the 2017 qualifying class during the review process. Finally, a special thanks to our VBI leadership for their continued support of our students and the production of the VRN.

I am honored to have been elected to serve as Editor-in-Chief of this year’s VRN. Having read these insightful reviews and seen the quality of work produced by our graduate students I am excited for the continued success and growth of the Neuroscience Graduate Program and the Vanderbilt Brain Institute.

Your Editor-in-Chief,

[Signature]

Allyson Mallya
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 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 founding in 2009 in an effort to consolidate and recognize the hard work done by each class of Ph.D. qualifiers, and is published annually by the Institute.

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

Reprints of individual articles are available from the authors or on the website. Requests for permission to reprint material published in VRN should be made in writing and addressed to the attention of Journal Permissions, Vanderbilt Reviews Neuroscience, 6133 Medical Research Building III, Nashville, TN 37232. The request must include a citation of the exact material that will be reprinted and specific information about where it will be used. One must receive written permission from the authors whose work will be reused. All copyrights are held by the Authors.

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Francis Cambronero
Randy Golovin
OUTREACH +
EDUCATION

A Message from the Interim Director of the Vanderbilt Institute

Dr. Lee Limbird, former Chair of the Department of Pharmacology, told me that the role of graduate school was “to learn to overcome the fear of failure”. In the nearly three decades that have passed since I first heard those words, I often reflect upon how failure represents an integral part of success, not only in graduate school, but also in any endeavor which one might choose to pursue. Albert Einstein was quoted as saying, “failure is success in progress”, yet somewhere along the way, we begin to associate failure with defeat, rather than opportunity. Somewhere along the way, we see failure as a monster, rather than a mentor.

For doctoral students in the Vanderbilt Training Program in Neuroscience, the admission to candidacy represents a critical first step on a much longer road to personal development and scientific training. Part of this process requires publication of a review in a student’s research area in Vanderbilt Reviews Neuroscience (VRN), which can be enjoyed within these pages. These early successes are the products of hard work, preparation, persistence and confidence, but also may have been punctuated by failures in the classroom, the library or the laboratory. These are not the last failures that neuroscience graduate students will experience. As Winston Churchill stated, “Success is not final, failure is not fatal: the only thing that counts and is forever is your courage to continue”.

May your year be filled with the kinds of failure that lead to exciting discoveries, new opportunities and scientific surprises.

Ronald B. Emeson, Ph.D.
Joel G. Hardman Professor of Pharmacology, Biochemistry, Molecular Physiology & Biophysics and Psychiatry & Behavioral Sciences
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A Message from the Neuroscience Program Director of Graduate Studies

Dear Readers,

It has been another successful and exciting year for the Neuroscience Graduate Program! With Ron Emeson taking the helm for Mark Wallace, the program has continued to flourish and a variety of changes have been made that benefit the students. Many of these developments are regarding funding and typically go unnoticed by the students, but they create a more equitable and balanced system for everyone. Ron has consistently been a champion of equal treatment for all students and we are all very grateful for the battles he’s fought on behalf of the program.

We are also excited to find out who the new VBI director will be and look forward to that announcement in the coming months. With the new director will certainly come a variety of new initiatives and changes that will reinvigorate the VBI and our graduate program.

It has been another successful year of recruiting new students. We admitted 6 students through the direct admit route and accepted 10 from the IGP and 2 MSTPs. As usual, they represent the cream of the crop and are from a wide variety backgrounds and locations. I look forward to working with all of them!

As always, our curriculum continues to evolve, with substantial input from the students. Our Fundamentals of Neuroscience courses have changed a bit this year, as they do each year, with some new lecturers and slightly revised curriculum. In addition, the Fundamentals II course (8340) will be undergoing substantial reorganization next year with a new course director, Thilo Womelsdorf. Thilo has a very interesting and innovative plan for revising the course and I look forward to its implementation! We are also revising the fall section of 8325 to focus on “rigor and reproducibility.” The new course will incorporate a substantial amount of statistics, giving all of our students a solid foundation in methods of data analysis.

The remarkable scientific achievements, the bold leadership and the commitment to service by our students never ceases to amaze me. I am always very impressed by the scholarly reviews written by our students for their Qualifying Exam and published in the VRN. These reviews typically serve as a springboard for further high quality publications based on their thesis research. This year, 15 of our students successfully defended their thesis, many having been recognized for their contributions with grants and awards. 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 (thank you to Ally Mallya for this edition), which they founded. Thank You All for your commitment to the program! It is a privilege to serve as the Director of Graduate Studies for such a fantastic group of students!

Sincerely,

Bruce Carter, Ph.D.
Associate Director for Education and Training, Vanderbilt Brain Institute
Director of Graduate Studies in Neuroscience
Professor of Biochemistry
OUTREACH +
EDUCATION

An Update from the Neuroscience President

Dear Vanderbilt Neuroscience Community,

It was my pleasure and an honor to serve as President of the Neuroscience Student Organization this year. In operation since 2000, the purpose of this organization is to support the professional development and success of the neuroscience graduate students, to promote community engagement and public outreach, and to encourage work-life balance among the graduate students. We are able to achieve these goals only through the hard work of the NSO student leadership and with the support of the VBI administrative team.

I would like to congratulate the neuroscience graduate students who passed their qualifying exams this year and transitioned into doctoral candidacy. Their qualifying exam review papers, published here, reflect the impressive conceptual and technical diversity of the ongoing research in our neuroscience community. Special thanks are due to Ally Mallya, Francis Cambronero, and Randy Golovin for editing and producing this edition of Vanderbilt Reviews Neuroscience. I would also like to thank our academic committee (Sierra Palumbos, Lauryn Luderman, and Collins Opoku-Baah) for their work preparing students for their qualifying exams.

The VBI and NSO had many outstanding accomplishments this year. The work supported by our outreach committee (Jacob Ruden, Salma Omer, and Francis Cambronero) this year demonstrates the continued commitment of the VBI and the NSO to meaningful community engagement. Jacob, Salma, and Francis spearheaded our outreach efforts, including the annual Brain Blast event, new classroom-based neuroscience lectures and hands-on brain dissections at Nashville Metro Public Schools, and a number of public talks. Sahana Nagabhushan Kalburgi organized and executed this year’s VBI Retreat at the beautiful Schermerhorn Symphony Center. Highlights of the day included the keynote address by Dr. Ramesh Balasubramaniam (Professor of Cognitive and Information Sciences at the University of California) and new faculty talks by Miriam Lense, Melissa Duff, Carrie Jones, and Vivian Gama. Sean Moran and Greg Salimando played vital roles in promoting the work-life balance and wellness efforts of the NSO by hosting several excellent NSO socials and the VBI Spring Picnic.

This year we welcomed the new Barlow Family Director of the Vanderbilt Brain Institute, Lisa Monteggia, to Vanderbilt. We are thrilled to have her join our community and look excitedly to the future of the VBI under her leadership. I would be remiss if I did not also specifically acknowledge and thank Ron Emeson for his exceptional service to the Vanderbilt Brain Institute during his time as Interim Director and now as Associate Director of the VBI. The entire NSO and VBI community joins me in extending a heartfelt thank you to Ron for his dedication to the VBI and to its students. Finally, I would like to underscore the invaluable contributions of our administrative staff, not least Roz Johnson and Beth Sims, to the neuroscience graduate program. Their commitment to the students and to the program forms the foundation for our successes.

I hope you join me in celebrating these accomplishments and the many more to come this year!

Gabriella DiCarlo
NSO President 2017-2018
Brain Blast | Community Outreach

Community outreach is a major focus of the Vanderbilt Brain Institute (VBI). The VBI Outreach Committee, made up of Ph.D. students and faculty from the VBI, plans and coordinates free outreach events ranging from interactive learning activities to seminars and lectures aimed at engaging the greater Nashville and Tennessee populations. This year, Ph.D. students Francis Cambronero, Salma Omer, and Jacob Ruden served as the Neuroscience Student Organization Outreach Coordinators, and Dr. Rebecca Ihrie served as the Faculty Chair of the Outreach Committee.

The VBI Outreach Committee’s signature annual event is Brain Blast, held during Brain Awareness Week, which is a worldwide celebration of the brain. This year’s event, Brain Blast 2018, was a resounding success. The event was held on a beautiful and sunny Saturday - March 17th, 2018 - at the Martin Professional Development Center. Nearly 500 kindergarten to 8th grade students and their families visited over 15 exciting booths to learn about bike safety from the Vanderbilt University Police Department, hold hissing Madagascar giant cockroaches, dissect various animal brains and eyeballs, extract DNA from strawberries, and more! A guest appearance from Mr. Commodore wearing his very own monogrammed lab coat made Brain Blast 2018 that much more special. We cannot wait to see what Brain Blast 2019 will have in store.

The VBI Outreach Committee also coordinated sheep brain dissections for local middle school students throughout the school year. “The students were ecstatic about being involved in the sheep brain dissections. They were particularly excited to directly apply what they had learned in class about the brain and nervous system to the dissections,” explained Salma Omer, the main organizer of these events. “The dissections are a great way to offer middle school students a hands-on learning experience about the mammalian brain.” The Outreach Committee also planned a neuroscience-themed day for summer campers at CampVandy, a new event that is likely to become an annual tradition.

In addition to youth-targeted events, the VBI hosted “get the facts” presentations and discussions about Alzheimer’s Disease at different Nashville Public Libraries, as well as a series of neuroscience lectures at the Osher Lifelong -- Learning Institute for the older adults in the community.
**Input-specific modulation of Reward Circuity by mGlu receptors**

**Allyson Mallya, Graduate Student**

The nucleus accumbens (NAc) is a hub for integrating excitatory inputs from different brain regions to direct reward- and motivation-related behaviors. Maladaptive synaptic changes and reorganization of neural circuits in the NAc are thought to contribute to motivational disorders and drug abuse. Although metabotropic glutamate (mGlu) receptors are known to play a principal role in modulating these synapses, how mGlu receptors regulate distinct NAc afferents was not understood -- until now.

Brandon Turner, Ph.D., while a neuroscience graduate student in the lab of Brad Grueter, Ph.D., Assistant Professor of Anesthesiology, dissected the modulatory impact of Group I mGlu receptors (mGlu1 and mGlu3) on different inputs to the NAc shell (NAcSh), specifically from the prefrontal cortex (PFC) and the medio-dorsal thalamus (MDT). In a report published in *Neuropsychopharmacology*, Turner and colleagues used a combination of whole-cell patch clamp electrophysiology, optogenetics, and pharmacology in transgenic mice to demonstrate that mGlu receptors differentially regulate NAcSh synapses based both on afferent origin and cell type.

The authors first demonstrated that both PFC and MDT neurons form connections with NAc medium spiny neurons (MSNs). To do so, they performed electrophysiology on acute brain slices from mice that had been infected with a virus to induce expression of channelrhodopsin (ChR2) in excitatory neurons in the PFC and MDT. Activation of neurons from each region individually, using blue light to stimulate ChR2, evoked excitatory synaptic activity onto both dopamine receptor type-1 (D1)- and type 2 (D2)-expressing MSNs, which canonically promote reward seeking and aversion behaviors, respectively.

They then examined how post-synaptic Group I mGlu receptors, which have an established role in triggering long-term depression (LTD) of excitatory signaling in the NAc, influence these NAcSh inputs. Low frequency stimulation was able to induce LTD in both D1 and D2 MSNs at PFC synapses, but only caused LTD in D1 MSNs at MDT synapses. MPEP, an antagonist of mGlu5 receptors, abolished LTD at MDT-D1 synapses but not in PFC-D1 or -D2 synapses. Conversely, LY367385, an antagonist of mGlu6 blocked LTD at all PFC-NAcSh synapses but not in MDT-D1 synapses, suggesting that mGlu receptors differentially function at PFC and MDT synapses.

Finally, Turner and colleagues examined whether cocaine, which has been shown to diminish mGlu LTD function in the NAc, impacts mGlu function at PFC and MDT synapses. Mice were subjected to a cocaine sensitization paradigm followed by a period of cocaine abstinence; LTD at specific synapses was then examined. Only mGlu5-dependent LTD at MDT-D1 synapses was impaired in cocaine-exposed mice; application of the mGlu5-selective positive allosteric modulator VU0409551 rescued LTD at these synapses.

Taken together, the data suggest differential regulation, both in input-specific and cell type-specific manners, of synaptic strength by mGlureceptors in the NAcSh. Targeting mGlu5 agonists may be therapeutically beneficial for alleviating maladaptive synaptic changes caused by cocaine and possibly other drugs of abuse. This study highlights the importance of further understanding synapse-specific plasticity and how doing so may guide novel treatment options for motivation and substance use disorders.

**Learn More:** Turner, B.D., Rook, J.R., Lindsley, C.W., Conn, P.J., Grueter, B.A. (2018). mGlu1 and mGlu5 modulate distinct excitatory inputs to the nucleus accumbens shell. *Neuropsychopharmacology, 43*,2075-2082.
**HIGHLIGHTS + BRIEFS**

**RESEARCH BRIEFS**

*Modulation of the BNST CRF Neuron Stress Response*

**Allyson Mallya, Graduate Student**

Stress is a major contributor to a variety of neuropsychiatric, mood, and affective disorders. The bed nucleus of the stria terminalis (BNST) plays a principal role in mediating the normal adaptive response to stress; however, chronic stress can cause maladaptive changes in BNST-dependent circuitry over time. The corticotropin releasing factor (CRF) system in the BNST is a key regulator of stress responses and contributes to negative affective behaviors.

A recent study from Tracy Fetterly, Ph.D., a neuroscience alumna from the lab of Danny Winder, Ph.D., explored how BNST CRF signaling is modulated following stress exposure.

Fetterly and colleagues first established that acute restraint stress increased the activation of BNST CRF neurons in mice. Administration of the α2A-adrenergic receptor (AR) agonist guanfacine, an inhibitor of certain CRF-dependent behaviors, prevented this increase, suggesting that the BNST CRF system is regulated in part by norepinephrine (NE). The authors then used electrophysiology to show that NE inhibits excitatory signaling to BNST CRF cells via α2A-AR.

Circuit mapping approaches demonstrated that both the parabrachial nucleus (PBN) and insular cortex send afferent projections to the BNST. Interestingly, only the PBN-BNST CRF synapses were sensitive to NE modulation. Using chemogenetic techniques, the authors demonstrated that activation of PBN neurons reduced BNST CRF cell activation in a manner similar to guanfacine.

Together, this article, published in *The Journal of Neuroscience*, establishes that stress enhances BNST CRF neuron activity and that α2A-AR activation can dampen this effect. Better understanding of how different these systems interact within the BNST to influence stress responses can guide future strategies for the treatment and prevention of related disorders.

Prefrontal Mediation of the Reading Network Predicts Intervention Response in Dyslexia

Randall Golovin, Graduate Student

Dyslexia, an impaired ability to read words despite intact comprehension and sufficient reading instruction, is the most common form of learning disorder. Even when the disorder is diagnosed early in life some people respond better to treatment than others. One place to start looking for individual differences are the reading network and frontoparietal control network (FPN) are two groups of interconnected brain regions that are activated by reading and executive processing, respectively. In psychiatric patients an interplay between cognitive systems and executive processing predicted learning outcomes. Katherine Aboud, a neuroscience graduate student, and her colleague working in Laurie Cutting’s laboratory thought that this work could extend to an interaction between the reading network and FPN explaining which dyslexic patients respond to treatment and which do not. The researchers suspected that a strong connection between the FPN and reading networks would be an important predictor of reading interventions for dyslexic patients. In an article recently published in Cortex, Aboud and her colleagues used functional magnetic resonance imaging (fMRI) to record brain activity from dyslexic and typically developing children before a reading intervention training looking for changes in the FPN and reading network that might explain differences in response to the treatment.

In order to elicit reading related areas in the brain, the authors showed children short words while in the fMRI scanner while recording the FPN and reading network brain regions. First, Aboud and colleagues looked at the connections in the reading network in isolation comparing three separate groups. 1) children who were typically developing (TD) 2) children with dyslexia who were responsive to treatment (DYS-R) 3) children with dyslexia who were unresponsive to treatment (DYS-NR). The scientists found that connections within the reading network were greatest in the DYS-R group suggesting that these individuals can deal with their dyslexia more effectively through a compensatory mechanism increasing activity in the reading network.

Next Aboud and colleagues explored how executive control areas of the FPN were connected to the reading network by seeing how well the activity of the FPN predicted the activation of the reading network. Similar to their first analysis the authors split the participants into TD, DYS-R and DYS-NR. The story with the FPN was different than the reading network with TD and DYS-R groups showing similar executive control over reading while the DYS-NR group showed reduced executive control. This means that dyslexic children who have executive function more similar to TD children can better utilize the reading interventions.

Overall, this paper by Aboud and colleagues made two important discoveries regarding the treatment strategies for individuals with dyslexia. First, people who respond to reading intervention have greater compensatory activity in their reading network. Second, dyslexic patients that respond to treatment have similar executive control over their reading network as TD children while dyslexic patients who do not respond to treatment have reduced executive control over their reading network. Together these findings lead to two important takeaways. One, not all children with dyslexia have the same underlying brain changes. Two, children with dyslexia should be treated with reading interventions and executive control training. This study tackled an important issue of dyslexia treatment and should pave the way to better treatments in the future.

ON THE COVER

This work was inspired by a recent publication, Aboud et al. (2018; *Human Brain Mapping*). This study applied a big-data framework and sophisticated regression strategy to map the brain volumetric trajectories, using over 5,000 MR images (ages 7-90). Another key highlight of the study was its potential illustration of changes in brain network organizational efficiency with age – i.e., structural covariance network (SCN). SCN reflects the brain structural underpinnings and correspondence of functional modalities; for example, brain regions, such as the lingual and middle occipital gyri and the superior and transverse temporal gyri, fall within the functionally-relevant visual and auditory networks, respectively (Alexander-Bloch et al., 2013; Evans, 2013; see also Guo et al., 2015). This artwork aimed to represent not only the coordinated maturational trends (local versus global changes) but also the coupling and de-coupling patterns of brain structure and function. Specifically, local brain structural changes fine-tune functional efficiency (such as identifying faces and objects and decoding words and numbers); yet, global brain structural refinement also strengthens the convergence and communication across brain functional networks (i.e., connectomics) to support the more cognitively taxing tasks, such as linguistic comprehension and theory of mind.

Main Reference (* indicates members of the Vanderbilt Brain Institute)


Supporting References


Chapter 5: Hemodynamics in Alzheimer’s Disease and Vascular Cognitive Impairment and Dementia

Francis Cambronerio

Hemodynamic impairment is one of the earliest detectable abnormalities in Alzheimer’s disease (AD)\(^4\) with well-documented cerebral blood flow (CBF) changes occurring in prodromal AD (i.e., mild cognitive impairment\(^5\) (MCI)) as well as in cognitively normal older adults at genetic risk for AD.\(^6\) Chronic cerebral hypoperfusion develops decades before cognitive decline and precedes the earliest detectable in vivo changes in amyloid-\(\beta\) (A\(\beta\)) accumulation, hypometabolism, and brain atrophy. These early vascular insufficiencies, including CBF dysfunction and blood brain barrier (BBB) breakdown, trigger the development of cerebrovascular pathology that drives cognitive impairment and may accelerate AD pathology. In fact, cerebrovascular disease (CVD) is the most common pathology to co-occur with AD,\(^7\) leading to possible additive and synergistic effects of both pathologies on cognitive impairment.\(^8\)\(^,\)\(^9\)

The clinical impact of hemodynamic impairment and CVD has been well characterized in vascular cognitive impairment and dementia (VCID). Among aging adults free of clinical dementia, CVD is frequently reported,\(^10\) particularly mild forms of cerebral small vessel disease (SVD), such as microinfracts detected at autopsy\(^11\) or diffuse white matter damage on magnetic resonance imaging (MRI).\(^12\)\(^,\)\(^13\)\(^,\)\(^14\) Plus, it is the second most common cause of dementia after AD.\(^6\)

Emerging evidence suggests there is considerable overlap between VCID and AD and that mixed pathologies account for most clinical dementia syndromes.\(^2\) Vascular lesions influence the presence of dementia in AD, both by lowering the threshold at which existing pathology will clinically present as overt dementia\(^16\) and potentially mediating or amplifying the severity of amyloid effects. Given the growing recognition of the link between cerebrovascular dysfunction and AD, it is important to understand how observed hemodynamic impairments contribute to worse vascular and neuronal health.

This chapter reviews the fundamentals of the cerebrovasculature, the tightly controlled mechanisms underlying CBF regulation, the early hemodynamic differences that emerge in AD and VCID, and how these changes are connected to dysfunction and damage of key neurovascular cells. The chapter also discusses how A\(\beta\) pathology in AD contributes to these pathways of damage in the cerebrovasculature and its surrounding tissue. Finally, the relevance of systemic contributions to cerebrovascular dysfunction and hemodynamic impairment, including vascular risk factors and cardiac function, is reviewed.

Section 1: An Introduction to the Cerebrovasculature

Blood flow control is especially critical to neuronal health and is tightly controlled by specific mechanisms at rest and under stress. The brain is one of the most metabolically demanding organs in the human body, consuming 20% of total oxygen and 25% of total glucose. It is incredibly sensitive to metabolic deficits, with hypoxia causing unconsciousness within seconds and irreversible neuronal damage within minutes.\(^12\)\(^,\)\(^19\) Tightly controlled systemic and CBF regulation mechanisms are thus required to ensure rapid and continuous delivery of vital substrates across a variety of conditions.

The body must respond to physical activity, external temperature, and other pertinent factors by adjusting blood flow delivery to stressed tissue for proper function. In basic fluid dynamics, flow results when a pressure difference is exerted over a given resistance. In humans, the heart’s left ventricular pump creates a large pressure difference every cardiac cycle that is exerted over vascular resistance segments to generate blood flow, analogous to an electrical circuit. The primary determinant of this vascular resistance to flow is vessel diameter, wherein smaller diameters increase resistance...
and reduce blood flow. However, predictive models of hemodynamics are further complicated by real-life vessel properties, including vessel wall compliance that dampens fluctuations in pressure and diverse vessel architecture that contributes to irregularities in flow patterns. Vessel tortuosity, non-smooth vascular walls, irregular arterial geometry, and branching patterns all contribute to variabilities in vascular resistance (and thus blood flow) throughout the vasculature.

Influential works like Duvernay et al (1981) have described the complexity of the vascular system of the human brain in detail, and it is important to underscore that vascular architecture varies more than neuronal circuitry. Primary feeding arteries in the neck join near the base of the skull to form the Circle of Willis, a structure that supports collateral circulation. There is considerable variation in Circle of Willis anatomy across humans, with only 21% of individuals possessing a completely patent structure on post-mortem examination and 36 to 42% on in vivo magnetic resonance angiography. The major arteries of the Circle of Willis give rise to progressively smaller pial arteries, which run along the surface of the brain in the pia-arachnoid space until they penetrate the brain parenchyma as intracerebral arterioles and begin associating with astrocytes and neurons. Pial arteries are structurally distinct from the microcirculation in several important ways. First, pial arteries are supported by collateral networks and innervated by perivascular nerves critical for regulation of cerebrovascular tone. In contrast, downstream arterioles are typically long and unbranched, making their flow territories more susceptible to damage from occlusion. Both pial arteries and intracerebral arterioles are surrounded by smooth muscle cell layers that further ensure greater basal tone. As parenchymal arterioles successively branch into capillaries, the smooth muscle cell layer gradually disappears and vascular tone is supplemented by pericyte contractile cells that wrap around capillaries. At this level of the BBB, vital substrates may be extracted from the blood and toxic metabolites may be cleared (e.g., carbon dioxide) via the selective diffusion barrier at tight junctions between endothelial cells. Blood flow to these capillary beds is primarily driven by smooth muscle cells, which locally control arteriole diameter and help amplify the pressure wave over the entire arterial tree. Thus, the complex and intricate structure of the human cerebrovasculature ensures adequate blood flow delivery to tissue by regulating vascular resistance through a variety of structural and cellular properties.

Section 2: Mechanisms of Hemodynamic Regulation

The cerebral circulatory system is controlled by distinct homeostatic mechanisms that ensure stable blood flow delivery in the face of fluctuating central perfusion pressures (i.e., cerebral autoregulation, commonly referred to as mechanoregulation) and further adapt blood flow to meet the metabolic demands of activated neurons (i.e., functional hyperemia or neurovascular coupling). Arterioles regulate CBF via adjacent smooth muscle cells, which induce local contractions in response to a variety of mechanical and chemical stimuli. Depending on how the smooth muscle membrane is polarized, it will either relax (hyperpolarization) or contract (depolarization) to control arteriole diameter and thus flow. This smooth muscle cell activity is regulated by mechanical stimuli and complex signaling cascades involving a variety of neurovascular cells. For example, neurons, endothelial cells, and astrocytes produce vasoactive substances known to regulate smooth muscle cells. Ultimately, vascular smooth muscle integrates a myriad of vasoactive and vasoconstrictive signals to regulate CBF, including signals propagated through passive diffusion, ligand-gated ion channels, and voltage-gated ion channels.

Cerebrovascular autoregulation mechanisms work at rest and throughout postural changes to protect delicate brain vasculature from changes in central blood pressure. Autoregulation ensures that the microvasculature receives a uniform level of CBF regardless of arterial blood pressure by narrowing arterioles in high-pressure states and dilating arterioles in low-pressure states. Although incompletely understood, cerebral autoregulation mechanisms are thought to occur in response to transmural blood pressure forces and linear shear stress (i.e., increased blood-flow velocity) that stimulate stretching of the vascular smooth muscle to maintain arteriole wall tension. Changes in arteriolar wall tension trigger biochemical signaling pathways in smooth muscle cells and the endothelium that work to modulate contractility. In contrast, neurovascular coupling is stimulated by vasoactive metabolites produced during neural activity. Neurovascular coupling involves well-characterized signaling pathways with contributions from astrocytic endfeet, interneurons, and retrograde endothelium signaling that work to regulate smooth muscle cell tone. The neurovascular coupling mechanism determines the hemodynamic response function, which is the physiological foundation for
CANDIDATE
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functional MRI. The initiators and mediators of neurovascular coupling are thought to be located at the capillary bed, and pericytes have recently been implicated as a candidate initiator of the functional MRI signal.

In addition to resting and functional mechanisms of CBF regulation, cerebrovascular reactivity (CVR) mechanisms (i.e., chemoregulation) play a critical role during physiological stress to clear carbon dioxide waste from the blood stream. This mechanism is the basis of the hypercapnic response often used in neuroimaging paradigms to assess arteriolar health. It is defined by the ability of the cerebrovasculature to increase CBF in response to arterial carbon dioxide changes (i.e., cerebrovascular reserve capacity). CVR mechanisms regulate CBF changes during hypercapnia via myogenic partial pressure-sensing mechanisms mediated by pH changes in the extracellular fluid and the formation of nitric oxide.

Although there is still conflicting evidence surrounding the underlying mechanisms and overlap between cerebral autoregulation, neurovascular coupling, and chemoregulation, these CBF regulation pathways are thought to be the most predominant and fast-acting. Despite well-documented sympathetic innervation of intracerebral arteries, the role of neurogenic regulation remains controversial, with some evidence suggesting it is beneficial in conditions of severe ischemia. With advancing age, these predominant resting and dynamic CBF control mechanisms may be increasingly less effective. Emerging evidence suggests that the quality of hemodynamics and perfusion appear to be compromised in both typical aging as well as disease, including AD and CVD, such as VCID. Such compromise ultimately places the brain at risk for oligemia, ischemic damage, and a cascade of pathological changes associated with vascular and neuronal injury.

Section 3: Patterns of Hemodynamic Impairment in Aging and Dementia

Although brain structure, activity, and cognitive abilities all undergo patterned changes with normal aging, some of the earliest patterned changes to emerge are in blood flow. Chronic hypoperfusion is promoted by aging and comorbidities, including hypertension, obesity, and diabetes. Early CBF studies demonstrated global and regional reductions in the aging brain, particularly in limbic or association cortices. Given that blood-oxygen-level-dependent MRI signals may be compromised in CVD due to altered neurovascular coupling and baseline CBF, more recent, reproducible studies have relied on arterial spin labeling MRI quantification of CBF. These MRI studies have revealed more complex, bidirectional regional CBF patterns in aging. Although global CBF is still reduced, there are increases in regional CBF, which may provide support for a functional compensation theory (cf. Cabeza et al., 2002). Furthermore, flow asymmetry is inversely related to memory performance in cognitively normal older adults. These findings suggest that in addition to global decreases in CBF, typical aging likely involves the bilateral recruitment of certain regions for new functional roles, perhaps to offset the effects of emerging reductions in brain activity and related CBF.

Unlike structural tissue measures, drivers of age-related changes in CBF remain harder to isolate and are largely unclear. Nevertheless, there are clear patterns of hypop- and hyperperfusion across the brain. Decreased regional CBF in older adults is likely intimately linked to subtle neuronal dysfunction due to oligemia and age-related neurodegenerative factors (e.g., reduced neuronal number, size, and activity, or synaptic density changes), while increased regional CBF may indicate neuronal compensation or pathological overactivation (i.e., hyperactivity that emerges in brain regions typically affected in AD). In addition, it is very likely that a lifetime burden of systemic vascular risk factors contributes to vascular dysfunction and compromised blood delivery, particularly when cerebral autoregulation mechanisms begin to fail in conditions like hypertension (during which higher perfusion pressures are needed to maintain the same level of CBF). Subtle changes in cerebral autoregulation may make the brain more susceptible to central hemodynamic changes.

There is consistent evidence of hemodynamic changes in VCID and AD, but both diseases appear to have pronounced CBF disturbances beyond typical aging with neuropathological overlap. Although previous Doppler-based CBF studies indicated that the severity of disturbances in cerebral hemodynamics may be higher in VCID compared to AD, both VCID and AD demonstrate CBF reductions relative to cognitively normal older adults, but it has been harder to distinguish CBF patterns in VCID and AD based on more recent arterial spin labeling MRI studies. Interestingly, both groups also have elevated pulsatility indices compared to cognitively normal controls, but patients with VCID demonstrate relatively higher pulsatility indices compared to AD. Blood flow pulsatility can be damaging to the
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microcirculation, contributing to vascular remodeling, increasing resistance, and failing cerebrovascular reserve over time. Accordingly, significant reductions in CVR have been found in VCIID compared to cognitive controls.6,26 However, similar to arterial spin labeling MRI CBF studies, there are minimal detectable differences in CVR between VCIID and AD,3,6,26 perhaps because of the neuropathological co-occurrence of these two diseases.

In AD, CBF patterns distinct from normal aging emerge early on, before tissue atrophy, and most noticeably as global hypoperfusion, which eventually correlates with cognitive impairment.6 Compared to cognitive controls, there is unique hypoperfusion of lateral temporoparietal and medial parietal regions, including precuneus and posterior cingulate cortex, in AD.6,66,67 Interestingly, hypoperfusion in parietal areas predicts conversion from MCI to AD.68 The earliest stages of AD are also associated with distinct patterns of regional hyperperfusion, most prominently in the hippocampus and basal ganglia,21 suggesting compensatory or pathological elevation of neural activity. Flow territory asymmetry (defined as how much an individual’s CBF pattern deviates from the more symmetrical group pattern) is positively correlated with memory performance in MCI but not cognitively normal older adults.21 This observation indicates that flow asymmetry may develop as a protective mechanism in early, prodromal AD to recruit additional resources for tissue operating at maximal cerebrovascular reserve capacity.

Emerging MRI studies of CVR in AD support the notion that cerebrovascular capacity may also be impaired in the earliest disease stages. In addition to hypoperfusion, CVR seems to be among the earliest neuroimaging markers of AD.22 While significant CVR reductions have been found in AD, the largest CVR impairments appear to be in bilateral frontal cortices.23 CVR is a measure of how well vessels respond to changes in arterial carbon dioxide and more accurately reflects myogenic function (i.e., smooth muscle cell contractility) than neurovascular coupling, which is dependent on neuroactive substrate release into the tissue. Although the hemodynamic response function is thought to be driven by neuronal-derived signals in healthy states (e.g., classical nitric oxide signal transduction), its physiological basis in AD may rely more heavily on alternative mechanisms when neurovascular coupling begins to fail and blood-oxygen-level-dependent MRI signals become more unreliable measures of underlying neuronal activity.24 The effectiveness of any given hemodynamic regulatory mechanism relies heavily on the integrity of its cellular components and tightly coupled molecular signaling pathways. Alterations in CBF in both VCIID and AD likely reflect microvascular dysfunction that may be driven by declines in capillary health, endothelial dysfunction, and other neurovascular unit cell problems. These eventual patterns of hypoperfusion will drive damage to the immediate vascular tissue and surrounding parenchyma.

Section 4: Hemodynamic Contributions to Vascular Injury and Neurodegeneration

Once hemodynamic impairments develop, they contribute to worse brain health primarily through ischemic and hemorrhagic injury. Subtle CBF reductions may result in oligemia, which promotes suboptimal neuronal activity due to deprivation of vital ions and nutrients and pathological protein homeostasis. However, more significant CBF reductions lead to ischemia, resulting in hypoxia-triggered oxidative-nitrosative stress and neuroinflammation damage pathways. Less common forms of hemorrhagic damage stemming from microbleeds and blood toxicity promote similar damage pathways secondary to disruption of the cellular architecture.22 The ultimate activation of apoptotic and necrotic cascade pathways drive neuronal damage and clinically detectable cerebrovascular lesions in the parenchyma, seen on MRI as cerebral SVD. These cerebrovascular lesions contribute directly to worse neuronal health, neurodegeneration, and subsequent cognitive impairment. Original definitions of vascular dementia focused on diffuse arteriosclerotic changes,24,29 including large vessel disease. However, a more recent reconceptualization of VCIID expands to all forms of cerebrovascular changes that affect cognition, including cerebral SVD, offering a more sophisticated perspective on cerebrovascular underpinnings of cognitive impairment and dementia.30,31

From a neuropathological perspective, cerebral SVD encompasses a variety of degenerative changes affecting small arterioles and microvessels of the brain, primarily driven by arteriolosclerosis, cerebral amyloid angiopathy, microaneurysms, and microvascular degeneration markers, such as fibrinoid necrosis and lipohyalinosis (i.e. arteriolar wall disorganization characterized by thickening and narrowing of small vessels). These pathologies are mostly observed in subcortical brain areas (e.g., basal ganglia and white matter) and lead to lacunar infarcts, hemorrhages, and axonal loss. Interestingly, recent evidence suggests the observance of even a few microinfarcts at autopsy implies the presence of hundreds, suggesting a substantial whole-brain burden of SVD capable of driving neurologic dysfunction.21
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Clinically, the term cerebral SVD is also used to describe larger parenchymal damage easily detected on MRI. Conventional MRI markers of SVD include white matter abnormalities, cerebral microbleeds, and lacunar infarcts, which have been shown to exist in up to 85% of aging adults and associate with cognitive impairment and dementia. White matter abnormalities appear as hyperintensities on T2 fluid-attenuated inversion recovery MRI. While presumably of vascular origin, such as ischemia, microhemorrhages, and damage to small blood vessel walls, they can be of varying pathogenesis, including gliosis, breaches in the cerebrospinal fluid (CSF) and brain barrier, or myelin sheath loss and deformation. Cerebral microbleeds result from impaired small vessel integrity, mainly attributed to either hypertensive vasculopathy or cerebral amyloid angiopathy. Lacunar infarcts are small (<15 mm in diameter), caused by ischemia, and located in the territory of deep penetrating arteries. While not a common MRI marker of SVD, perivascular spaces are visible fluid-filled spaces adjacent to cerebral vessels thought to reflect tissue damage due to impairments in perivascular drainage, particularly driven by hypertension and abnormal arterial pulsatility in the basal ganglia. Traditionally, perivascular spaces have been considered benign with limited clinical significance. However, recent work by our group suggests perivascular spaces not only correlate strongly with information processing speed and executive function, they also appear to have more robust associations with cognition than other SVD markers.

In addition to ischemic and hemorrhagic damage, hemodynamic insufficiencies also promote damage to the BBB and neurovascular unit. As described above, the BBB is critical for both providing the neuron with blood-derived nutrients and minerals and protecting the neuron from toxic blood components that can cause diffuse oxidative damage and outright cell death (e.g., exposure to red blood cells). BBB permeability increases in advanced aging and has become an intense focus of recent AD research. The neurotoxic consequences of BBB breakdown include endothelial metabolic dysfunction, pericyte dysfunction, and hypoperfusion-hypoxia. Taken together, current evidence suggests hypoperfusion, SVD, and BBB breakdown are closely related in cognitive impairment and dementia. Going forward, both typical and abnormal brain aging studies will benefit from examining how early vascular changes fit into the larger pathological cascade to understand drivers of disease and how neuronal health is ultimately affected.

Section 5: Interactions of AD Pathology with Vascular Dysfunction

Cerebrovascular pathology has overlapping and even possibly synergistic effects with amyloid pathology, the pathological hallmark of AD. In autopsy series, cerebrovascular pathology reflects the second most common pathology after AD and the most common pathology to co-occur with AD. Furthermore, vascular dysfunction is common in AD. Both patients with prodromal AD and transgenic AD mouse models have accelerated BBB breakdown, along with focal microcirculatory changes, such as string vessels, reductions in capillary density, rises in endothelial pinocytosis, decreases in mitochondrial content, accumulation of collagen and perlecans in the basement membrane, and loss of tight junctions. These prominent AD-related vascular changes have intimate interactions with Aβ metabolism, as described below.

Accumulating evidence suggests cerebrovascular compromise may contribute to impairments in amyloid transport across various clearance pathways. Aβ is eliminated from extracellular spaces primarily via exchange across the BBB. However, a smaller percentage of Aβ clearance is also supported by interstitial fluid (ISF) bulk flow pathways, including traditional perivascular clearance (i.e., ISF drainage through basement membranes of smooth muscle cells in the capillary and arterial walls) and lymphatic perivascular clearance (i.e., CSF influx into the periarterial space and CSF-ISF exchange in the parenchyma). Thus, small vessel injury likely affects Aβ accumulation across various perivascular and BBB clearance pathways. Molecular evidence further indicates that impairments in Aβ clearance receptor expression, including increased receptors for advanced glycation end products implicated in Aβ influx into the brain and decreased lipoprotein receptor-related protein receptors involved in Aβ efflux into the circulation. In addition to clearance impairments, hypoperfusion in AD may stimulate diffuse AD pathology, including Aβ deposition, tau hyperphosphorylation, and reductions in autophagy. Chronic hypoperfusion purportedly promotes Aβ pathology through aberrant amyloid precursor protein processing (e.g., increases in β-secretase/γ-secretase amyloidogenic activity) and local Aβ release from degenerating smooth muscle cells and endothelial cells.

Evidence supporting Aβ-vascular interactions suggests pathological changes in CBF may occur along shared pathways with Aβ pathology and compromise the structural and functional integrity of cell types involved in hemodynamic regulation. CBF is reduced in inferior parietal cortices in participants with amyloid-positive MCI compared to normal...
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cognition\textsuperscript{107} These reductions are further extended to inferior temporal and precuneus regions in participants with amyloid-positive AD, tracking with typical AD pathology progression\textsuperscript{107}. On a cellular level, Aβ pathology may cause changes in cell types of the neurovascular unit, including endothelial and pericyte damage and basement membrane thickening. These microvascular cellular changes are accompanied by functional reductions in glucose transport across the BBB and reductions in blood flow shear stress critical for maintaining vascular endothelial cell health and survival.\textsuperscript{41,42} Aβ likely disrupts upstream vessel reactivity mechanisms and promotes oxidative stress that both inhibits vasodilation\textsuperscript{108-110} and contributes to the degeneration of smooth muscle cells, a common feature of AD. In this manner, Aβ can be a potent disrupter of hemodynamic regulation at all levels of the arterial tree.

The vascular structural abnormalities in AD brains described above likely increase vascular resistance and contribute to hypercontractility and hypoperfusion. Indeed, recent studies show increased pulsatility and resistance indices in AD.\textsuperscript{111} Moreover, functional deficits in neurovascular unit signaling may contribute to neurovascular uncoupling, which is well-documented in AD.\textsuperscript{112,113} Age- and AD-related alterations in cerebrovascular structure and functional reactivity may reduce the efficacy of cerebral autoregulation,\textsuperscript{63,114} especially when vascular risk factors are present.\textsuperscript{26} In this manner, the brain may be more vulnerable to damage over time, particularly from exposure to subtle age-related alterations in cardiovascular hemodynamics.

Section 6: Cardiac Contributions to Cerebral Hemodynamics

As the central blood pump of the human body, the heart plays an essential role in hemodynamic regulation and determines critical characteristics of initial blood flow, including volume, pressure, and pulsatility. The brain consumes 15% of cardiac output, a measure of forward stroke volume, despite only accounting for 2% of total body weight. Like dementia, cardiovascular dysfunction becomes increasingly common with advancing age, with 28% of older adults experiencing some form of prevalent cardiovascular disease (e.g., coronary heart disease, angina, heart attack, heart failure, or peripheral artery disease).\textsuperscript{115} Furthermore, as many as 97% of older adults also have at least one key cardiovascular risk factor, including high blood pressure, diabetes, high cholesterol, or smoking, all of which are associated with a higher burden of subclinical cardiovascular dysfunction.\textsuperscript{116} Thus, alterations in heart health can have implications for brain health, particularly in older adults with more vulnerable vascular systems.

Severe cardiac dysfunction in the form of clinical heart failure is a known risk factor for cognitive impairment\textsuperscript{117-119} and incident clinical dementia,\textsuperscript{120,121} likely through microvascular dysfunction and reduced CBF.\textsuperscript{122,123} However, evidence from our group\textsuperscript{124,126} and others\textsuperscript{27,128} suggests even subclinical reductions in cardiac output are associated with worse cognitive outcomes. Lower cardiac output is associated with worse executive function in aging cardiac patients,\textsuperscript{124} worse information processing speed and executive function in middle-age and older community dwelling adults,\textsuperscript{125,128} and a higher risk of incident clinical dementia and AD in community-dwelling aging adults.\textsuperscript{126} We have recently shown that cardiac strain, an early MRI marker of changes in contractility of the myocardial fibers, relates to episodic memory and language performance.\textsuperscript{129} Nearly all of these findings persist when excluding participants with heart failure, prevalent cardiovascular disease, or arrhythmias\textsuperscript{25,126,129} suggesting subclinical changes in cardiac function with advancing age correspond to cognition with regional vulnerability implicating the temporal lobes.

Cardiac hemodynamics may relate to worse cognition through disturbances of cerebral hemodynamics and subsequent neurodegeneration. Indeed, our research has shown that lower cardiac output is associated with smaller cerebral gray matter volume,\textsuperscript{125} greater subcortical white matter damage,\textsuperscript{120} and CBF reductions as recently reported for the first time in humans.\textsuperscript{131} While these latter findings were anatomically global, the most robust effects were regionally specific to the temporal lobes. The temporal lobes may be particularly vulnerable to systemic hypoperfusion given they are especially sensitive to hypoxia\textsuperscript{132} and the earliest neuroanatomical location of BBB degeneration in aging and MCI.\textsuperscript{133} Theoretical models linking more severe cardiac dysfunction to AD support reduced systemic perfusion as a primary driver of reduced CBF (as we have recently observed in a subclinical setting\textsuperscript{21}) but further implicate compensatory neurohormonal activation, inflammation, and microvascular dysfunction as secondary contributors to impaired CBF.\textsuperscript{124} These more mild, prolonged damage pathways are likely larger drivers of cerebrovascular and neuronal dysfunction in subclinical cardiac dysfunction rather than outright ischemia and neuronal failure. Chronic but subtle reductions in cardiac output likely results in oligemia with a gradual metabolic energy crisis for neurons, leading to inflammation, oxidative stress, and dysfunction of enzymes and protein synthesis. It is important to note that although cerebral autoregula-
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tion mechanisms generally serve to maintain a constant CBF level, the accumulation of vascular risk factors and vascular damage with older age may compromise the integrity of these mechanisms.26 Thus, in the setting of advanced aging with a lifetime of vascular burden exposure, subtle changes in systemic blood flow and pressure may exert a larger adverse impact on brain health, including the development or progression of SVD, AD pathology, and neurodegeneration.

While a direct pathway between cardiovascular changes and brain health is plausible, alternatively, cardiovascular dysfunction may relate to worse brain health as a consequence of emerging neuropathology (i.e., a brain to heart pathway). Several reports indicate that evolving pathology and neurodegeneration in the AD brain may drive disruptions of autonomic control circuits responsible for heart rate and blood pressure control. Indeed, autonomic dysfunction has been repeatedly described in AD patients,133-139 including impaired cardiovagal parasympathetic function140,141 and vasomotor sympathetic dysfunction.142 Although Braak and Braak staging criteria do not include evaluation of the brainstem, a central regulator of autonomic function, its early involvement in AD suggests emerging relevance.133 AD pathophysiology may cause a disruption of essential brainstem circuitry responsible for cardiovascular control, upstream autonomic control centers responsible for integrating peripheral signals and regulating the brainstem144,145 (e.g., hypothalamus and insula), and cortical modulators of these autonomic circuits.146 Moreover, cholinergic signaling pathways associated with cerebral and peripheral nerve dysfunction135,147 may be particularly vulnerable in AD. Ultimately, varied abnormalities in brain structures and networks subserving autonomic regulation may impair cardiovascular function in AD, accounting for previously reported connections between cardiovascular function and abnormal brain changes.124-126,129-131

It is also possible that the link between cardiovascular and brain dysfunction is an epiphenomenon. In support of this hypothesis, pleiotropy between AD and cardiovascular risk-associated genes has been increasingly established. For example, variants in the presenilin-1 gene, the same gene associated with early-onset AD, have been reported in idiopathic dilated cardiomyopathy.148 These variants appear to reduce protein expression of the presenilin 1 protein, likely compromising its direct roles in calcium signaling149,150 and excitation-contraction coupling148 rather than amyloid processing. More recent genetic studies have indicated that the polygenetic component of AD is also enriched for cardiovascular risk factors, particularly lipid-associated factors potentially linked to BBB damage and pathological cholesterol metabolism in the brain.151,152 In addition to a common genetic profile affecting the heart and brain, studies of cardiovascular protein abnormalities in AD have further demonstrated a common molecular profile. In AD patients, biochemically similar Aβ deposits have been shown to co-exist in the heart and brain, and myocardial Aβ deposits appear to contribute to early diastolic dysfunction, as defined by impaired left ventricular relaxation.153 Although cardiovascular amyloid is common in advanced age,154 cardiovascular Aβ40 and Aβ42 expression is particularly increased in AD.155 The possibility that Aβ aggregates may directly drive cardiomyocyte defects is strengthened by findings that myocardial Aβ oligomers promote changes in calcium homeostasis that likely mediate cardiomyocyte toxicity and contractile dysfunction in idiopathic dilated cardiomyopathy.148 Future genetic, biochemical, and molecular studies will likely continue to shed light on the shared mechanisms underlying declines in heart and brain health, as well as whether these declines occur independently or through a shared pathway.

Section 7: Conclusions

The clinical diagnosis of dementia is increasingly recognized to be driven by pathophysiological processes decades in the making.155 Early hemodynamic changes likely contribute to and reflect frequently co-occurring cerebrovascular pathology in AD. Although vascular impairments are known to contribute independently and through Aβ interactions to neuronal dysfunction, further research is needed to disentangle additive, mediating, or synergistic effects. Regardless, emerging basic science studies and recent clinical trial results support the notion that preventing and treating vascular disease will reduce incident MCI and dementia, both by reducing the clinical significance of existing AD pathology and decelerating the deposition of emerging AD and vascular pathology. To gain a more comprehensive understanding of VCI and AD pathogenesis, emerging measures of hemodynamic compromise (e.g., time-course of response156), regional distribution patterns, and other key players in cerebral metabolism (e.g., glucose utilization, oxidative metabolism) should be further examined across the AD clinical spectrum and incorporated into a larger framework for understanding vascular dysfunction in dementia.
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The Neural Chronometry of Attentional Bias: Evidence for Early and Late Stages of Selective Attentional Processing

Resh Gupta

Abstract
Although our visual system is constantly overwhelmed by streams of incoming information, selective attention allows us to focus on relevant information and filter out irrelevant information. In all humans, selective attention is powerfully biased to threat-related information. Threat-related biases in selective attention have been demonstrated using visual search tasks and rapid serial visual presentation. Threat-related biases can become problematic when they shift to clinical levels, as in anxiety disorders. Studies using behavioral tasks such as the dot-probe and emotional Stroop have been essential in establishing the existence of threat-related bias in anxious individuals. However, in these studies, commonly used measures such as reaction time do not clarify the process by which attention is deployed to threatening stimuli. Fortunately, the event-related potential (ERP) technique, with its millisecond resolution, allows for the continuous measurement of attentional processing across time. This review explores the neural chronometry of attentional bias to threat by analyzing results from studies utilizing ERPs in anxious populations. These analyses provide the background to investigate whether interventions such as Attention Bias Modification Treatment and Mindfulness-Based Cognitive Therapy are capable of shifting early and late ERP markers of attentional bias to threat and whether the intervention-induced modifications of these ERP markers are associated with improved anxiety symptoms.

Keywords: attentional bias, threat, anxiety disorders, event-related potentials, dot-probe, emotional Stroop, Attention Bias Modification Treatment, Mindfulness-Based Cognitive Therapy

Introduction: Attentional Bias
The visual system, like all sensory systems, is constantly bombarded by streams of information competing for awareness. However, only a small amount of the information available on the retina can be processed and used in the control of behavior. Fortunately, competition can be resolved through selective attention, which is the ability to focus on information currently relevant to behavior while filtering out irrelevant information. In all humans, selective attention is powerfully biased to threat-related information, since mammals evolved in environments where dangers constantly threatened survival and reproductive advantage. Attentional bias to threat is defined as the preferential allocation of attention to threatening stimuli over emotionally neutral stimuli.

According to cognitive and neural theories of emotion, attentional processing of innate threat stimuli (e.g. angry facial expressions) is prioritized over neutral stimuli. Studies using rapid serial visual presentation paradigms to investigate the effects of emotional face stimuli on attentional blink have found enhanced detection of faces associated with threat or danger compared with neutral and happy faces. Similarly, in visual search tasks, fear-relevant pictures were found more quickly than fear-irrelevant ones, and threatening faces guided
visual search more efficiently than happy or neutral faces. These findings support the proposal that, when there is competition for attentional resources, threat stimuli are given higher priority in processing compared with non-threatening stimuli. Importantly, selection for awareness is influenced by the significance of stimuli to the observer. For example, participants fearful of snakes but not spiders (or vice versa) showed facilitated search for the feared objects but did not differ from controls in search for nonfeared fear-relevant or fear-irrelevant targets.

Although threat-related biases are important for survival, they can become detrimental when they shift to clinical levels, as seen in anxiety disorders. Anxiety disorders, such as generalized anxiety disorder (GAD), social anxiety disorder (SAD), and specific phobia are associated with muscle tension and vigilance in preparation for future danger and cautious or avoidant behaviors. The attentional system of anxious individuals may be distinctively sensitive to and biased in favor of threat-related stimuli in the environment, and it has been demonstrated that attentional bias towards threat among anxious populations is a relatively robust phenomenon. Threat-related biases may play an important role in maintaining anxiety states, as anxious individuals would be more likely to detect minor potential threats in their environment, thus intensifying their anxious mood state.

Theories suggest that anxious individuals are prone to biases at specific stages of information processing. Information processing is commonly divided into two stages: automatic and strategic. Automatic processing occurs without intent, control, or awareness and is capacity free, which means it does not require cognitive capacity. Strategic processing is intentional, controllable, capacity-limited, and dependent on awareness. Williams et al. proposed that the attentional system of anxious individuals is abnormally sensitive to threat-related stimuli, and these individuals tend to direct their attention toward threatening information during early, automatic stages of processing. Abnormalities in the threat-detection mechanism of anxious individuals would, therefore, lead them to adopt a hypervigilant mode toward threat. In contrast, Foa and Kozak proposed that inhibition of detailed processing of threatening information is the core deficit in anxiety, which is reflected in avoidance of threatening stimuli. According to this view, threat-related biases in anxiety are confined to later stages of processing. A vigilance-avoidance model proposed to reconcile these views states that anxious individuals tend to direct their attention toward threat during early, automatic stages of processing, whereas during later, more strategic stages of processing, they tend to direct their attention away from threat. Fox et al., however, suggest that anxiety has little impact on initial detection of threat but has a stronger effect in modulating the maintenance of attention on the source of threat. They have proposed that a delay in disengaging from threat stimuli might be the primary attentional difference between anxious and non-anxious individuals.

Anxiety disorders have thus provided an excellent model in which to study the components, or observable and measurable characteristics, of attentional bias to threat: facilitated attention to threat, difficulty disengaging attention away from threat, and attentional avoidance of threat. Facilitated attention, or hypervigilance, refers to the relative ease or speed with which attention is drawn to a threat stimulus. The amygdala, critically
involved in the processing of fear-related information and expression of fear-related behavior, is likely involved\textsuperscript{10}. Difficulty in disengaging refers to the degree to which a threat stimulus captures attention and impairs switching attention from the threat to another stimulus. Prefrontal cortical (PFC) regions including the orbitofrontal cortex and functionally related structures such as the anterior cingulate cortex are likely involved, as they serve a regulatory purpose and can down-regulate emotion-relevant limbic structures\textsuperscript{10}. In attentional avoidance, attention is preferentially allocated towards locations opposite the location of the threat cue\textsuperscript{10}. PFC-centered activity mediates emotion regulation, which is linked with attentional avoidance\textsuperscript{10}.

It has been shown that attentional biases depend on both automatic and strategic processing\textsuperscript{10}. However, determining whether the components of attentional bias may be tied to specific stages of information processing\textsuperscript{10} is essential for the development of interventions targeting these components. Understanding how attention is deployed to emotional stimuli\textsuperscript{21} in anxious populations can shed light on this issue. Behavioral studies have helped establish the existence of threat-related bias in anxious individuals\textsuperscript{8}, but commonly used measures such as reaction time (RT) provide an indirect measure of attentional processing\textsuperscript{22} and can be confounded by post-perceptual processes such as motor responses and decision making\textsuperscript{23,24}. Fortunately, the use of event-related potentials (ERPs) allows for the examination of the time course of attention to threat with millisecond resolution\textsuperscript{1,25}. In order to elucidate the neural chronometry of threat-related attentional bias, this review focuses on behavioral paradigms and findings and, subsequently, delves into physiological studies using the ERP technique.

**Behavioral Measurement of Attentional Bias**

Behavioral studies of attentional bias to threat vary greatly. Stimuli used consist of threatening and non-threatening words, pictures\textsuperscript{9}, or happy, angry, and neutral faces. Subliminal exposure conditions, which preclude conscious awareness, or supraliminal exposure conditions, which allow access to awareness\textsuperscript{9}, can be used. Anxious populations used also vary. Some studies focus on a specific anxiety disorder, such as SAD. Subjects may be diagnosed with clinical anxiety or have non-clinical anxiety evidenced by scores on anxiety questionnaires such as the State-Trait Anxiety Inventory (STAI)\textsuperscript{9,26}. The STAI measures conscious awareness at two extremes of anxiety affect: state anxiety and trait anxiety. State anxiety is defined as fear, nervousness, discomfort, and the arousal of the autonomic nervous system induced temporarily by situations perceived as dangerous (e.g., how a person is feeling at the time of a perceived threat). Trait anxiety is defined as a relatively enduring disposition to feel stress, worry, and discomfort\textsuperscript{27}. Participants may be recruited based on trait or state scores. Most importantly, different paradigms reflecting the operation of attentional processes\textsuperscript{9} have been used. Four of these paradigms are as follows:

**Emotional Stroop task:** The emotional Stroop task, a modified version of the original Stroop paradigm\textsuperscript{28}, utilizes a similar mechanism as the original with one important change: threatening or neutral words are presented in varying colors and participants are required to report the color of the word while ignoring its semantic content. Biased attention for threat is inferred when color naming is slower or less accurate for threat-
Emotional spatial cueing paradigm: In the emotional spatial cueing paradigm developed by Fox et al., participants focus on a fixation mark at the center of the screen. Subsequently, a cue (e.g., a threatening or neutral stimulus) appears in one of two locations, and after its disappearance, a target appears in one of the two locations. Participants are required to quickly and accurately indicate the identity or location of the target. Valid trials are those in which the target appears at the location that was previously cued, while invalid trials are those in which the target appears at the location not previously cued. Attentional bias to threat is indicated by faster responses on valid threat-cued trials relative to neutral-cued trials, and slower responses on invalidly threat-cued trials relative to neutral-cued trials.

Visual search task: In a visual search task, participants must locate and respond to a target stimulus within an array of distracting, non-target stimuli. The target could be threat-related (e.g., a picture of a snake) surrounded by non-threatening distractors (e.g., pictures of flowers), or the target could be neutral (e.g., a picture of a mushroom) surrounded by threatening distractors (e.g., pictures of spiders). Attentional bias is demonstrated when participants are faster to respond to threat-related target stimuli amongst neutral distractors or are slower at detecting a neutral target stimulus amongst threat-related distractors.

Dot-probe paradigm: In the dot-probe task, created by MacLeod and colleagues, two visual (i.e., word or picture) stimuli, called cues, are briefly presented. One cue is commonly threat-related and the other is neutral. After the cue stimuli disappear, a small probe, or target, appears in the location of one of the cues. Participants must quickly and accurately respond to the location or identity of the probe. Faster responses to probes are observed when they occur in the attended rather than unattended location. Thus, participants displaying attentional bias will typically demonstrate faster responses to probes that appear in the location of a threatening compared to neutral stimulus. The dot-probe has been deemed by some as the gold-standard in attentional bias research because participants respond to the neutral dot-probe instead of the word or picture stimuli themselves, thus, eliminating response bias interpretations (though, see Kappenman, Farrens, et al., 2014 for a disagreement).

Behavioral Findings in Anxious Populations: A meta-analysis of 172 studies (N = 2,263 anxious, N = 1,768 non-anxious) conducted by Bar-Haim et al. revealed that anxious, but not non-anxious, populations display a significant attentional bias to threat, and the magnitude of the bias is similar across a variety of populations with anxiety. Additionally, anxious populations but not control subjects demonstrate significant threat-related bias in emotional Stroop, dot-probe, and emotional spatial cueing paradigms. However, behavioral studies investigating the different components and time course of anxiety-related attentional bias are conflicting. Mogg et al. manipulated the exposure duration of word pairs (100 milliseconds (ms), 500 ms, 1500 ms) in a dot-probe task with non-clinical anxiety patients. Higher levels of state anxiety were associated with faster response latencies for probes that replaced threat words rather than neutral words, reflecting attentional vigilance for threat. However, the bias was not significantly affected by the exposure duration of the word stimuli. Years later, Mogg et al. conducted another dot-probe study using pictorial stimuli presented for
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500 ms or 1500 ms. Compared to low trait anxious participants, high trait anxious participants were more vigilant for high-threat scenes at 500 ms, but showed no attentional bias at 1500 ms\(^3\). Clearly, behavioral measures such as RT are not sensitive enough to clarify the times at which individuals are hypervigilant or avoidant toward threat-related stimuli. To further examine the chronometry of attentional bias, ERPs have been used to examine how the allocation of attention unfolds over the course of a trial\(^3\).

**Physiological Measurement of Attentional Bias**

Using the superior temporal resolution of ERPs, researchers have investigated the neural correlates and timing related to the processing of emotional stimuli, cues, and targets in attentional bias paradigms. Differential processing of threatening stimuli is inferred when one or more ERP features, such as amplitude, latency, and scalp distribution, are modified for threat-related but not neutral stimuli. Amplitudes are generally assumed to signify the degree or intensity of the engagement of cognitive processes, and latencies are thought to measure the time course of stages of processing\(^3\). Peak amplitude and peak latency are measured at the maximum point in a defined time window, but mean amplitude can also be measured by taking the average voltage over a specified measurement window\(^3\). Each ERP component has a distinctive scalp distribution that reflects the location of the patch of cortex in which it was originally generated; however, it is difficult to determine the exact location of the neural generator source simply by examining the distribution of voltage over the scalp\(^3\). Most ERP components relevant to attentional bias are modulated by emotional stimuli and spatial attention\(^3\). The early C1, P1, N1, N170, P2, N2, and N2pc components and the late P3 ERP component have been analyzed in the literature. A brief description of each follows.

**C1:** The C1 ERP is not labeled with a P or an N because its polarity can vary. It is thought to originate in the primary visual cortex (V1), and onsets before any other visual ERP component\(^3\). It is the first ERP component triggered by the appearance of a stimulus in the visual field and is thought to be pre-attentive and independent of spatial attention\(^3,35,36\). The C1 wave typically onsets 40-60 ms poststimulus and peaks 80-100 ms poststimulus\(^3\).

**P1:** The C1 is followed by the P1, which is typically the first major visual ERP component putatively originating in the extrastriate visual cortex. It is largest at lateral occipital electrode sites and typically onsets 60-90 ms poststimulus with a peak between 100 and 130 ms\(^3\). Allocation of attention to stimuli leads to an increased P1 amplitude\(^3\).

**N1:** The P1 is followed by the N1, which consists of several visual N1 subcomponents that sum together to form the N1 peak. The earliest N1 subcomponent peaks 100-150 ms poststimulus at anterior electrode sites. There appear to be at least two posterior N1 components that peak 150-200 ms poststimulus; one arising from the parietal cortex and another arising from the lateral occipital cortex. Although all three subcomponents are influenced by spatial attention, discrimination of attended stimuli specifically enhances the lateral occipital N1 subcomponent\(^3\).

**N170:** One component of the N1 wave is the N170, which typically peaks around 170 ms after stimulus onset and is largest over ventral areas of the visual cortex\(^3\). It has been shown that faces elicit a more negative
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potential than non-face stimuli at lateral occipital electrode sites, especially over the right hemisphere, with a peak at approximately 170 ms\textsuperscript{34,37}. The N170 is generally thought to be a face-specific ERP component\textsuperscript{37}, and has also been shown to be sensitive to emotional expressions\textsuperscript{38}.

**P2:** A distinct P2 wave follows the N1 at anterior and central scalp sites, but at posterior sites, the P2 is often difficult to distinguish from the overlapping N1, N2, and P3 waves\textsuperscript{34}. The P2 component occurring over occipital locations may reflect a distributed network of visual processing areas sensitive to threat-related stimuli, and may indicate more elaborate sustained perceptual processing\textsuperscript{39–41}.

**N2:** The second major negative peak is the N2, and it is made up of several subcomponents including the N2a, N2b, and N2c\textsuperscript{34}. The posterior N2 (N2c)\textsuperscript{34} for visual stimuli has been associated with discrimination and classification of stimuli. The peak latency of the component varies as a function of the difficulty of discrimination and correlates with the timing of discriminative behavioral responses\textsuperscript{42}. The N2 has been observed in studies involving processing of emotional content\textsuperscript{31}.

**N2pc:** The N2pc (N2-posterior-contralateral) is a subcomponent of the posterior N2, typically occurring between 200 and 300 ms. It is observed at posterior scalp sites contralateral to an attended object. The N2pc has been useful for determining whether attention has been covertly directed to a given object and for assessing the time course of attentional orienting\textsuperscript{34}. Studies have shown that task-irrelevant fearful faces elicit an N2pc, supporting that threat-related stimuli can bias the distribution of spatial attention\textsuperscript{43,44}.

**P3:** The P3 peaked at 300 ms when it was first discovered\textsuperscript{45}, but has since been found to peak anywhere between 350 and 600 ms\textsuperscript{34}. Although there is no clear consensus on which neural or cognitive processes are reflected by the P3 wave\textsuperscript{34}, the effect of various manipulations on P3 amplitude and latency have been well explored\textsuperscript{34}. The P3 amplitude is influenced by the amount of attention allocated to a stimulus\textsuperscript{46}; the amplitude is larger when subjects devote more effort to a task. Additionally, the hallmark of the P3 wave is its sensitivity to target probability; the amplitude gets larger as the target probability gets smaller. The P3 latency is thought to reflect the time required to categorize a stimulus and is insensitive to subsequent response-related processes\textsuperscript{34}. Distinguishable ERP components in the time range of the P3 wave include the frontally maximal P3a component and a parietally maximal P3b component. Although both subcomponents are elicited by unpredictable, infrequent changes in the stimuli, the P3b component is present only when these changes are task-relevant. Researchers generally use the term “P3” to refer to the P3b component\textsuperscript{34}.

To clarify the neural chronometry of attentional bias to threat, literature was reviewed to determine if and how features of these ERP components are modified to emotional and threatening but not neutral stimuli. The following findings, presented by paradigm, focus on healthy controls and anxious populations, as anxiety disorders provide an excellent model in which to study attentional bias to threat.

**Dot-Probe Studies**

**C1:** Eldar et al. measured attentional bias toward threat, neutral, and positive face stimuli and found that anxious participants had a more distinct C1 negativity compared to non-anxious participants solely in threat conditions, where angry-neutral face pairs were presented. This suggests that, at the level of V1, anxious indi-
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Individuals have perturbations in early, pre-attentive threat processing 35. Similarly, when Pourtois et al. conducted a dot-probe task with healthy participants using emotional-neutral pairs of face stimuli, they observed an enhanced negative C1 component with striate origin for fearful compared to happy faces. This provides further evidence that, as early as 90 ms post-stimulus presentation, V1 activity is enhanced by fear cues 37. However, Santesso et al. found that the C1 elicited by face stimuli was not modulated by emotional valence or attention in healthy adults. This result implies that the C1 may not be a consistent measure of either early emotion-related neural activation arising from the V1 or selective attention towards emotionally significant stimuli 48.

**P1:** Using high and low trait anxiety groups, Fox et al. observed enhanced P1 amplitudes to targets following presentation of angry expressions. However, this effect was not modulated by trait anxiety levels 34. In contrast, in their study with anxious and non-anxious groups, Eldar and colleagues failed to find threat-related differences in the P1 component locked to the target processing phase of the dot-probe task 35. In healthy adults, Pourtois et al. found that the lateral occipital P1 component occurring approximately 130 ms poststimulus selectively increased when the probe replaced a fearful face compared to a neutral face. This effect was not found for upright happy faces or inverted fearful faces 37. Using angry-neutral and happy-neutral face pairs, Santesso et al. found that the P1 amplitude was larger for validly cued probes following angry faces than invalid probes, confirming that threatening cues can modulate spatial attention in healthy adults. They also observed larger P1 amplitudes for invalidly cued probes following happy faces compared to validly cued probes or validly cued neutral faces. This result suggests that, in happy-neutral face pairs, attention was directed toward the relatively more threatening stimulus within the visual field (the neutral face) 48.

Several dot-probe studies have specifically focused on individuals with SAD. These individuals have a persistent fear of one or more social or performance situations in which they are exposed to unfamiliar people or to possible scrutiny by others 8. According to Helfinstein et al., individuals with high social anxiety showed higher mean P1 amplitudes to face display onsets (all composed of angry-neutral face pairs) compared to individuals with low social anxiety. This enhanced P1 may indicate increased sensory processing of faces in individuals with high social anxiety 39. Similarly, in participants reporting high fear of negative evaluation (FNE), Rossignol et al. observed increased P1 amplitudes in response to pairs of faces, irrespective of the emotional expression included in the pair. Additionally, while non-anxious subjects showed similar responses to targets following neutral or emotional faces, individuals with high FNE showed enhanced P1 amplitudes to targets replacing emotional faces. These results indicate that, in social anxiety, an early hypervigilance to face stimuli occurs 40. Mueller et al. demonstrated that, compared to controls, SAD participants had potentiated P1 amplitudes to angry-neutral versus happy-neutral face pairs and decreased P1 amplitudes to probes replacing emotional (angry and happy) versus neutral faces. These results suggest an early hypervigilance to angry faces in SAD and reduced visual processing of emotionally salient locations at later stages of information processing, potentially indicating attentional avoidance 24.

**N1:** Helfinstein et al. showed individuals with low and high social anxiety either a neutral or socially threatening prime word prior to emotional face display (composed of angry-neutral face pairs) and probe
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occurrence. Regardless of anxiety group, participants showed a trend towards greater N1 mean amplitudes for face display on threat prime trials compared to neutral prime trials. However, the low social anxiety group displayed more negative N1 mean amplitudes to face display onset than the high social anxiety group. These results suggest differential processing of the face displays between the groups, regardless of prime condition\(^9\). In healthy adults, Santesso et al. demonstrated that the N1 amplitude elicited by the probe was not modulated by cue validity\(^{48}\).

N170: In a study conducted by Rossignol et al. with high and low social anxiety groups, the N170 did not appear as sensitive to anxiety levels nor the emotional load of presented face pairs\(^{50}\). Similarly, in healthy adults, Santesso et al. found that the N170 to face stimuli was not modulated by emotional valence\(^{48}\).

P2: Eldar et al. demonstrated that, compared to non-anxious individuals, anxious individuals displayed enhanced occipital P2 amplitudes in response to face displays, regardless of whether the facial emotion was angry, happy, or neutral. These results suggest that modulation of the P2 amplitude serves as an indicator of the attentional commitment to processing of facial emotional expressions\(^{35}\). Studies focusing on SAD offer conflicting results. In a study by Rossignol et al., individuals with high FNE displayed enhanced P2 amplitudes in response to angry-neutral compared to fear-neutral face pairs. These results suggest that in SAD, angry faces are more salient than fearful faces, making it difficult for individuals to disengage attention from these stimuli\(^{49}\). Indeed, Helfinstein et al. showed that, compared to individuals with low social anxiety, individuals with high social anxiety displayed a trend toward more positive P2 mean amplitudes to face display onset. Additionally, regardless of anxiety group, participants showed a trend towards smaller P2 mean amplitudes in response to face display on threat prime trials compared to neutral prime trials. As mentioned above, in this study, primes were presented prior to face display and probe occurrence\(^9\).

N2pc: In a large sample of participants with individual differences in anxiety, Kappenman et al. observed that, following the onset of threat-neutral image pairs, the N2pc was elicited to the location of the threatening stimulus, reflecting a shift of covert visual attention in the direction of the threatening image. However, the N2pc was not correlated with trait anxiety, thus failing to provide a meaningful index of individual differences in anxiety in the dot-probe task\(^3\). Kappenman and colleagues conducted a dot-probe study with healthy participants and also found an initial shift of attention to threat-related stimuli reflected by the N2pc\(^{25}\). Fox et al. observed that angry expressions elicited an enhanced N2pc, but only in participants reporting high levels of trait anxiety. These results suggest that threat-related stimuli capture early attention in anxiety, reflecting a form of early attentional bias\(^{44}\).

Emotional Stroop Studies

P1: Using high and low trait anxious populations, Li et al. observed an enhancement of occipital P1 amplitudes to threat words that was more prominent the higher the level of trait anxiety. The authors interpreted this modulation as a signal of unconscious processing, as it was early and independent of whether word exposure was subliminal or supraliminal\(^{51}\). Sass et al. also demonstrated that, compared to a control group, an anxious arousal group showed an early processing bias evidenced by larger P1 amplitudes to emotionally arousing words; however, the P1 was specifically enhanced for pleasant stimuli, indicating preferential atten-
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ional to emotionally arousing and not threatening stimuli alone. Additionally, in the anxious arousal group, women had larger P1 amplitudes than men regardless of emotional content, evidencing greater early visual processing in females81.

Studies focusing on individuals with SAD are unclear. In an experiment conducted by Kolassa et al., social phobics, spider phobics and controls had to identify either the color or the emotional quality of angry, happy, or neutral schematic faces. These faces depicted prototypical features of threat. Social phobics displayed generally larger P1 amplitudes compared to spider phobics and non-phobic controls, but no general threat advantage for angry faces was found. These results indicate the presence of hypervigilance in phobic participants52. However, another study by Kolassa et al. demonstrated that the early visual P1 amplitude did not differ between social phobic, spider phobic, and non-phobic groups in response to angry faces; although, in all groups, the P1 amplitude was larger in response to emotional compared to neutral faces38.

N170: In the study by Kolassa et al., social phobics, spider phobics, and controls identified either the color (modified Stroop task) or the emotional quality (emotion identification task) of angry, happy, or neutral schematic faces. In all groups, emotional schematic faces led to larger N170 amplitudes than neutral schematic faces in the color identification task. This effect was even more pronounced in the emotion identification task, with angry schematic faces eliciting even larger N170 amplitudes than happy schematic faces82. In another study conducted by Kolassa and Miltner, socially phobic and non-phobic individuals identified either the gender (modified emotional Stroop task) or the expression of angry, happy, or neutral faces. Social phobics displayed larger N170 amplitudes over right temporo-parietal sites compared to controls when identifying the emotion of an angry face, and higher scores on the Social Phobia and Anxiety Inventory (SPAI)53 were associated with larger N170 amplitudes in response to angry faces. The expected emotional interference in patients with social phobia when identifying the gender of an angry face was not observed. With these results, the authors suggest that social phobics show abnormalities in early visual processing of angry faces88.

P2: Kolassa and Miltner demonstrated that social phobics showed no deviations from controls in P2 amplitudes to angry faces88. However, in healthy individuals, Thomas et al. found larger P2 amplitudes to threat words in the right compared to the left hemisphere. Although hemispheric differences have not been consistently identified, some data suggest that the right hemisphere is more involved than the left in processing negative emotional information54.

N2: In their study with anxious apprehension, anxious arousal, and control groups, Sass and colleagues observed enhanced N2 amplitudes to emotionally arousing words in the anxious apprehension group, reflecting an early processing bias for emotional words81.

P3: Using high and low trait anxiety groups, Li et al. found that the P3 amplitude was enhanced for threat versus neutral words, but the enhancement increased with higher trait anxiety only in the subliminal, not supraliminal, condition of the experiment. This suggests that non-conscious analysis of threat is intensified in individuals prone to anxiety, as is a later stage of threat processing subject to dynamic interactions between automatic and strategic influences81. Sass et al. demonstrated that anxious apprehension, anxious arousal and control groups displayed equivalent processing of emotional words as indexed by the P3 ampli-
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tude, with an enhanced P3 component associated with emotional arousal\textsuperscript{51}. In healthy adults, Thomas et al. also found larger P3 amplitudes for threat words, indicating more thorough or intense processing of these stimuli during higher level, controlled stages of cognition\textsuperscript{54}.

Other Experimental Paradigms

P1: Using a modified cue-target paradigm, Li et al. tested participants with high and low trait anxiety and determined that the P1 was modulated by threatening information contained in pictorial cues, occurring as early as 90 ms poststimulus in the contralateral hemisphere. In the high anxious group, the occipitoparietal P1 amplitude was enhanced for valid threatening cues relative to valid nonthreatening cues, reflecting vigilance to the location of threat. In the low anxious group, however, the P1 amplitude tended to be enhanced on threatening invalid trials, reflecting avoidance from the location of threat. These results suggest that an individual’s anxiety level determines the mechanism by which attentional bias to peripheral threatening stimuli modulates visual inputs in early processing stages\textsuperscript{55}. Bar-Haim et al. employed an attention-shifting paradigm with high and low trait anxiety groups. Faces with neutral, angry, fearful, sad, or happy expressions were presented singly at fixation. Participants fixated on the face cue and then discriminated a target shape that appeared randomly above, below, to the left, or right of the fixated face. Compared to the low-anxious group, the high-anxious group displayed significantly faster P1 latencies to face cues and slower responses to targets regardless of the emotion expressed by the face cue. These results suggest that high-anxious participants exhibit increased attentional dwelling on the face cues, thus interfering with the speed of target discrimination\textsuperscript{51}. In another study with high and low trait anxiety groups conducted by Holmes et al., participants viewed a series of fearful faces randomly interspersed with neutral faces in one experimental block, and then viewed a series of happy faces randomly interspersed with neutral faces in a second experimental block. Participants had to detect immediate repetitions of stimuli. Compared to the low trait anxiety group, the high trait anxiety group displayed an enhanced early visual P1 component over occipital locations to fearful faces. This enhancement may reflect a neural basis for attentional vigilance for threat-related material in anxiety, reflecting the output of a “threat evaluation system” whose threshold for activation is lower in high-trait anxious compared to low-trait anxious individuals. Heightened activation of this system could lead to stronger representations and attentional processing of threat-related stimuli\textsuperscript{56}.

N1: In the aforementioned attention-shifting paradigm conducted by Bar-Haim et al., the high trait anxiety group displayed faster N1 latencies to emotional face cues. Coupled with the finding of slower response times to targets in this group, results suggest that high-anxious participants exhibit increased attentional dwelling on the face cues, thus interfering with the speed of target discrimination\textsuperscript{51}.

P2: In the aforementioned attention-shifting paradigm conducted by Bar-Haim et al., the high trait anxiety group displayed larger P2 amplitudes than low-anxious participants to angry face cues, suggesting that threat-related stimuli elicit greater mobilization of attentional resources in high-anxious participants\textsuperscript{51}.

P3: In the aforementioned attention-shifting paradigm with high and low trait-anxiety groups conducted by Bar-Haim et al., no between-group difference was observed in the characteristics of the P3 component to target stimuli\textsuperscript{51}. 

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Oddball Paradigm: Few attentional bias studies have included an oddball paradigm within their design. The P3 component is most commonly studied within the framework of this paradigm, where infrequent stimuli elicit a much larger P3 wave than frequent stimuli\textsuperscript{34}. In a study by Mercado et al., individuals were selected based on their trait anxiety scores, and state anxiety levels were measured later in these participants. Emotionally positive, negative, and neutral images were presented visually over several minutes to anxious individuals. Concurrently, neutral auditory stimuli were presented in an oddball paradigm. Attention levels to the neutral auditory stimuli were assessed by observing the P2 amplitude, which the authors maintain is an index of auditory attention enhanced in the presence of unexpected auditory stimuli. Interestingly, only under conditions of high state anxiety, threatening context triggered an increase in attention to auditory stimuli, reflected by larger P2 amplitudes. These results suggest that high state-anxious individuals deploy specific hypervigilance under negative situations or threatening contexts\textsuperscript{57}. Rossignol et al. employed a more traditional oddball paradigm with low and high trait anxiety groups. Participants had to quickly detect deviant happy or fearful faces amongst a train of standard stimuli, which were neutral faces. The authors found that anxiety did not modify early perceptual ERPs including the P1, N1 or N170, nor the early attentional N2b component. However, later components were affected. High-anxious subjects exhibited significantly earlier P3b latencies to deviant faces and a decreased N3 component, reflecting a reduced ability to process the emotional content of faces. The authors propose that the earlier P3b observed in the high anxious group could be interpreted as a way to overcome the deficient emotional appraisal by a more salient conscious processing\textsuperscript{58}.

Conclusions and Future Directions

The reviewed studies highlight the usefulness of ERP methodology as a sensitive measure for the study of attentional bias and its chronometry\textsuperscript{41}. Certain ERP components are strongly and reliably modulated by threatening information at specific stages of attentional processing, making them useful markers of facilitated attention to threat, difficulty disengaging attention away from threat, or attentional avoidance of threat. To summarize the findings, more studies analyzing the C1 component are needed to validate that this very early, pre-attentive component is modulated by threat. The P1 appears to be most reliably modulated by threatening information and an individual’s anxiety level. In high-anxious populations, enhanced P1 amplitudes and faster P1 latencies to emotional cues and targets replacing threatening cues appear to reflect an early hypervigilance to threat-related information. Studies suggest that the N1 amplitude and latency are sensitive to anxiety levels and threatening cues, potentially reflecting increased attentional dwelling on these cues; however, more evidence is needed to support this conclusion. The N170 amplitude is modulated by angry faces in individuals with SAD, suggesting that social phobics show abnormalities in the early visual processing of these faces. Particularly in high-anxious participants, the P2 component appears to reflect mobilization of attentional resources to threat-related stimuli and difficulty disengaging attention from threatening stimuli. The N2pc seems to be involved in the initial, covert shift of attention to threat in healthy and anxious individuals. Finally, modulation of the P3 component appears to reflect more thorough, intensive processing of threat at later, controlled stages of cognition. By linking each ERP to the component of threat-related attentional bias it likely supports, a model of the neural chronometry of threat-related bias emerges (Figure 1). At very early, automatic stages of processing, facilitated attention toward threat, or hypervigilance, is reflected in
modulations of P1 and N1 features. Difficulty disengaging attention away from threat follows, reflected in modulations of the P2 component. At later, strategic stages of processing, attentional avoidance of threat is likely associated with modulations in P3 and subsequent ERP components.

In order to improve outcomes in anxious populations, it is essential to determine whether interventions such as ABMT and MBCT are capable of shifting early and late ERP markers of attentional bias to threat. Moreover, it is crucial to determine whether intervention-induced modification of these ERP markers is associated with improved anxiety symptoms.

**Figure 1. A proposed model of the neural chronometry of attentional bias to threat.** ERP markers identified in this review are linked to the components of attentional bias they most likely support. At early, automatic stages of processing, facilitated attention to threat occurs, and is associated with modulations of the P1 and N1 components. Difficulty disengaging attention away from threat follows, and is associated with modulations in the P2 component. At later, strategic stages of processing, attentional avoidance of threat occurs, and is associated with modulations in later ERP components such as the P3. In this image, negative voltages are plotted upward.

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Huntington’s Disease and DNA response/repair pathways to reduce oxidative stress

Piyush Joshi

Abstract

Huntington’s disease (HD) is a neurodegenerative disease which is characterized by unwanted chorea as well as behavioral and psychiatric disturbances which result from the death of neurons primarily in the caudate and putamen of the basal ganglia. HD is an inherited disease caused by the elongation of a trinucleotide CAG repeat on the short arm of chromosome 4p16.3 for which there is no cure. In HD, the Huntingtin (HTT) gene gains CAG repeats over many generations and over lifetimes due to a failure of cellular response and repair mechanisms. Manganese (Mn) homeostasis may underlie the pathological phenotypes in HD patients and Mn deficiency could lead to genomic instability, since Mn is a co-factor for many DNA replication and repair enzymes. Evidence that DNA repair mechanisms are impaired in HD may elucidate new therapeutic targets. This review discusses the role of Mn and oxidative stress in HD, particularly with regards to DNA response/repair pathways altered in HD and related neurodegenerative diseases.

Keywords: Huntington’s disease (HD), neurodegenerative disease, DNA damage response (DDR), DNA damage repair, oxidative stress, Manganese (Mn)

Huntington’s Disease

Huntington’s Disease (HD) is an autosomal dominant, age‐progressive neurodegenerative disease characterized by behavioral abnormalities, cognitive decline, psychological disturbances, and dyskinesia. It has a prevalence of 5–10 cases per 100,000 people among Caucasians, and its mean onset is 30–50 years of age, with a range of 2 to 85 years. The average lifespan of HD patients after diagnosis is 17–20 years, during which time the individual’s physiologic and behavioral symptoms progress, their dependency on caregivers increases, and they ultimately die most commonly by pneumonia. The characteristic symptoms of HD are primarily due to loss of GABAergic medium spiny neurons (MSNs), particularly in the striatum; however, neurons in the substantia nigra, globus pallidus, thalamus, subthalamic nucleus, subregions of the hypothalamus, and cortical layers 3, 5, and 6 are also vulnerable to cell death. There is currently no cure for HD; however, many therapeutic options are available for treating symptoms. Hyperkinesia, or chorea, is a motor symptom that is treated with agents that block or deplete dopamine receptors, such as neuroleptics and tetrabenazine, respectively. Medical and non-medical treatments are tailored for each individual, since symptoms differ between individuals and change over progression of disease.

HD is caused by expansion of a polyglutamine repeat near the N-terminus of the approximately 350kDa Huntingtin (HTT) protein on the short arm of the chromosome 4p16.3 on exon 1. Normal wild type HTT has unresolved functions, but appears to be critical for neuronal and synaptic development and embryogenesis. There is evidence that mutant HTT leads to gain of function as well as loss of function. Mutant HTT
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causes abnormal interactions in HD, driving mitochondrial dysfunction, oxidative stress (indicated by dysregulation of SOD1 oxidative stress gene\textsuperscript{11}), and excitotoxic events resulting in cell death. Studies with striatum and cortex of HD patients show a significant increase in DNA double stranded breaks as a result of increased oxidative stress\textsuperscript{12,13}. It has been shown that mutant HTT can gain additional CAG repeats over generations and lifetimes due to failure of cellular repair mechanisms. For this reason, HD in particular is associated with genomic instability since long GC-rich regions, such as the CAG expansion in mutant HTT, are susceptible to errors during replication. In addition to genomic instability, the polyglutamine repeat region of the HTT protein is particularly vulnerable to aggregation, which is a hallmark of HD\textsuperscript{14}.

An individual with normal HTT will have 6-26 CAG repeats within exon 1, but HD is associated with 40 repeats or more\textsuperscript{2}. HD in monoallelic and if an individual has more than 40 CAG repeats in either HTT allele, then they will develop disease as some point in their life\textsuperscript{14}. Furthermore, there is also an inverse correlation in HD between lengths of repeat and age of onset: the greater the number of repeats, the earlier the age of onset. However, age of onset of HD is highly variable between patients with the same repeat lengths and can differ by up to years and decades. This suggests that environmental factors may be playing in important role in determining the exact age of onset\textsuperscript{15,16}. This is supported by studies of monozygotic twins who differ in their age of onset by seven years\textsuperscript{17}. Also, it is worth noting that normal and mutant HTT are expressed in all tissues from birth and yet symptoms typically occur late in life. This would suggest that the presence of mutant HTT is not sufficient for pathology, but also requires age-related environmental stress\textsuperscript{18,19}. Identifying the environmental factors as well as the mechanism by which they contribute to age of onset may provide valuable insight for developing targeted therapeutics. Such environmental factors may include toxic exposures, nutritional status, and age-related environmental stress. A few environmental factors, including pollutants and heavy metals such as Mn, have been identified as potential contributors to HD pathology\textsuperscript{20-22}. This review illustrates connections between HD and Mn in the context of oxidative stress and DNA damage response and DNA repair pathways.

Heavy metals and Huntington’s disease

Previous studies have implicated iron (Fe), copper (Cu), and manganese (Mn) in the pathobiology of HD and alterations of these metals have been reported with HD. These metals accumulate in the brain and exhibit neurotoxic properties, likely accelerating neurodegeneration in HD. These metals are suggested to serve as environmental modifiers of age of onset since they can illicit neurotoxic effects independent of HD pathology\textsuperscript{23}. Furthermore, these heavy metals are important cofactors for enzymes that regulate cellular processes, but excessive metal ion concentrations can also result in oxidative stress, mitochondrial dysfunction, aggregation, and apoptosis. Accumulation of heavy metals in the brain by disturbance in metal homeostasis has been shown to lead to degeneration in the brain, specifically in the basal ganglia\textsuperscript{24,25}. Conversely, abnormally low concentrations of these metals are also detrimental to development and function\textsuperscript{26-30}. For instance, HD is associated with elevations in Fe and Cu levels but decreases in Mn in the corpus striatum\textsuperscript{22,31}. Reductions in the brain Mn is especially problematic as Mn is critical for the proper function of many proteins and enzymes, including manganese superoxide dismutase (MnSOD), arginase, glutamate synthase, and MRE-11, which have decreased activity in HD (Figure 1). Decreased Mn levels in HD striatal cell lines, mouse models, and patient brains illustrates a potential role for Mn as an environmental modifier of the pathophysiology of HD\textsuperscript{22,32-35}. 
Manganese, Huntington’s disease, and oxidative stress

Previous studies have implicated iron (Fe), copper (Cu), and manganese (Mn) in the pathobiology of HD and alterations of these metals have been reported with HD. These metals accumulate in the brain and exhibit Studies have found a significant reduction in Mn concentration in the HD cortex\textsuperscript{36,37}. However, there is evidence that Mn to accumulates in the globus pallidus and caudate nucleus: regions particularly susceptible regions to neurodegeneration in HD\textsuperscript{38,39}. This suggests a necessity of Mn for function of neurons in these regions. Furthermore, models of HD in vivo and in vitro show a decreased Mn-uptake phenotype, which suggests that there is Mn deficiency in HD\textsuperscript{40,41}. Alterations in the Mn levels in HD provides evidence for its role in neuronal health as well as HD pathology. Specifically, the lack of Mn homeostasis may underlie the pathological phenotypes in HD patients. For instance, a Mn deficiency could lead to genomic instability as Mn is a co-factor for DNA replication and repair enzymes (Figure 1). Reduced activity in these enzymes due to Mn deficiency, could contribute to mitochondrial dysfunction, excitotoxicity, and oxidative stress\textsuperscript{42-45}.

| Mre-11 | Function: Vital for repair of double-stranded DNA breaks  
Mn interaction: Binds Mn ions responsible for exonuclease activity  
Associated HD-phenotype: Reduced ATM activity |
|---|---|
| MnSOD (SOD2) | Function: Protects cells from ROS  
Mn interaction: Mn is necessary cofactor for MnSOD activity  
Associated HD-phenotype: Increased oxidative stress and reduction in mitochondrial function |
| p53 | Function: Regulates stress signals  
Mn interaction: Activated with exposure to Mn  
Associated HD-phenotype: Mutant HTT alters p53 functions |
| ATM | Function: Kinase that plays crucial role in double-stranded DNA break repair  
Mn interaction: Necessary cofactor for activity  
Associated HD-phenotype: Increased oxidative stress, DNA damage |
| Translesion polymerases | Function: Traverse DNA damage during replication  
Mn interaction: Mn enhances efficiency of translesion DNA synthesis  
Associated HD-phenotype: Increased oxidative stress |
| FAN1 | Function: DNA nuclease  
Mn interaction: Mn required for optimal activity of enzyme  
Associated HD-phenotype: Increased DNA damage |

**Figure 1:** Mn-dependent and Mn-responsive enzymes involved in oxidative stress, DNA damage response and repair pathways.

Mn toxicity has been shown to inhibit all four mitochondrial complexes of the electron transport chain via oxidative stress\textsuperscript{46}. In addition to mitochondrial toxicity due to elevated Mn, reduced Mn is also harmful since it alters the activity of protective MnSOD, which leaves the mitochondria vulnerable to damage. MnSOD is an antioxidant mitochondrial metalloenzyme which protects cells from reactive oxygen species (ROS) and death by converting superoxide ions into H$_2$O$_2$ and oxygen. MnSOD is critical for detoxifying ROS in the
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in the mitochondria and Mn is a necessary cofactor for its activity. Mn supplementation has been shown to increase MnSOD\textsuperscript{47}, and Mn deficiency leads to reduced oxygen uptake and dysmorphic elongation of mitochondria due to lack of ROS detoxification\textsuperscript{48}. P53 is also known to inhibit expression of MnSOD, which is consistent with the increased p53 levels and oxidative stress in HD\textsuperscript{49}. Thus, appropriate homeostatic regulation of Mn is needed to ensure proper cellular function and avoid toxicity.

In HD, the HTT gene gains repeats over many generations and over lifetimes due to a failure of cellular response and repair mechanisms. HD has been associated with genomic instability, along with other neurodegenerative disorders, and is inversely related to risk for cancer. HD patients have a six times decreased risk of cancer compared to siblings\textsuperscript{50}. A significant fraction of HD induced pluripotent stem cells (iPSCs) have increased genetic abnormalities compared to control iPSC lines during reprogramming, demonstrating an increase in genomic instability in HD\textsuperscript{51}. Many DNA response and repair enzymes are also Mn-dependent or Mn-responsive. For example, MRE11, an important part of DNA repair complex MRN, has a di-manganese binding pocket and Mn is necessary for its nuclease activity\textsuperscript{44,45}. The nuclease activity of MRE11 is vital for proper repair of double-stranded DNA breaks (DSBs) by homologous recombination. Reduced nuclease activity due to loss of Mn would result in stalled DSB repair as well as prolonged activation via phosphorylation of Ataxia Telangiectasia Mutated (ATM) resulting in increased DNA damage in HD. Furthermore, Mn has also been found to regulate activity of translesion DNA polymerase activity\textsuperscript{53}. These translesion DNA polymerases increase cellular tolerance for DNA damage by allowing specialized polymerases to traverse over DNA damage as opposed to repairing it during replication\textsuperscript{52,53}. Lower Mn concentrations lead to reduced activity of translesion DNA polymerases, which increases oxidative stress in HD\textsuperscript{54,55}. Collectively, these findings suggest Mn acts upstream of DNA response and DNA repair pathways in response to oxidative stress.

P53/ATM pathway in Huntington’s disease and manganese

P53 is a heavily studied pathway and is important for regulating all cellular stress signals, including DNA damage, oxidative stress, and other stressors that elicit a pro-survival or apoptotic transcriptional response\textsuperscript{56}. P53 has been implicated as a major player in the neurodegeneration of HD and is activated with exposure to Mn. HD patients have an accumulation of p53 in the brain that correlates to disease stage. Mutant HTT is thought to bind to p53 and alter its function\textsuperscript{57}. Cellular models of HD show increased phosphorylation and activation at serine 15 on p53\textsuperscript{50,58}. This increased activation of p53 occurs by the upstream kinase ATM\textsuperscript{49}, which has been implicated in abnormal activation and phosphorylation of p53\textsuperscript{50}. ATM is the canonical kinase for p53 and plays a crucial role in DSB repair pathway\textsuperscript{51,62}. P53 has been shown to be a metal binding protein that uses Zn\textsuperscript{2+} to maintain proper conformation\textsuperscript{63}; however, the ability of Mn to interact directly with p53 is not well established. In the context of DNA damage, ATM’s activity is increased in HD models due to auto-phosphorylation at serine 198\textsuperscript{64}. In the event of DSBs, ATM is converted into an active monomer that phosphorylates important downstream targets (e.g., gamma-H2AX, CHK2, p53) that regulate DNA repair, cell cycle arrest, and apoptosis. Mn is necessary for the in vitro enzyme activity of ATM, including phosphorylation of p53 at serine 15\textsuperscript{65,66}. The relationship between ATM and Mn is not well established, but these studies indicate that Mn may be necessary for ATM to function properly. Both p53 and ATM have been reported to upregulated in HD and inhibition of these protein rescues HD phenotype\textsuperscript{67,68}.

DNA response/repair pathways in neurodegenerative diseases

Thousands of DNA lesions occur per day in individual human cells. If these lesions are not repaired or repaired incorrectly, they can lead to complications in genome replication and transcription, which threaten the viability of that cell. DNA damage may be caused by DNA mismatches during DNA replication, DNA strand breaks caused by topoisomerases, hydrolytic reactions, non-enzymatic methylation, ROS, and
environmental toxic agents, such as heavy metals\textsuperscript{69,70}. To combat DNA damage, cells have established DNA-damage response (DDR) pathways to detect DNA lesions, signal, and promote repair (Figure 2)\textsuperscript{71-73}. Neurons are exposed to high levels of DNA damage and cannot recover easily due to the fact they are post mitotic. For this reason, DDR pathways have evolved to recognize damage through the activation of select kinase activity, such as ATM which activates downstream factors that mediate apoptosis or survival. The key DDR signaling components are ATM and Rad3-related protein (ATR), which are protein kinases that are activated by DSBs and replication protein A (RPA)-coated ssDNA\textsuperscript{74-76}.

Accumulation of DNA damage and impairment of DNA repair systems are involved in the pathogenesis of many neurodegenerative diseases, including Alzheimer’s (AD), Parkinson’s (PD), and HD\textsuperscript{77,78}. Neurons exhibit high respiration which is associated with increased ROS that may be damaging to mitochondrial and nuclear DNA\textsuperscript{79}. Defects in repair pathways (e.g., base excision repair and SSB repair) have been shown to trigger neuronal dysfunction and degeneration\textsuperscript{77,80}. Neurons are particularly susceptible to DNA damage as the same repair mechanisms in mitotic cells are not present. For instance, replication-associated DNA repair cannot occur in terminally differentiated post mitotic neurons\textsuperscript{81}. Furthermore, neurons rely heavily on transcription, and DNA damage can interfere with this process. The genomes of neurons are particularly vulnerable to oxidative damage due to an abundance of ROS generated during high O\textsubscript{2} consumption as well as weak anti-oxidant systems\textsuperscript{82}. This suggests that accumulation of DNA lesions might progressively deprive neurons of vital transcripts leading to apoptosis and cellular dysfunction\textsuperscript{83}. If DNA damage is extensive then DNA response pathways trigger senescence and/or apoptosis. To maintain DNA fidelity during the course of a postmitotic neuron’s lifetime, many DDRs with overlapping networks of independent DNA repair pathways have evolved to fight this constant challenge\textsuperscript{84}.

**Figure 2: Model for DNA damage response in the presence of DNA damage.** DNA damage by oxidative stress, environmental toxins, etc. can lead to activation of sensors, including Mre-11 which is part of the MRN complex. Sensors then activate transducers and effectors (e.g., ATM and p53), which activate repair proteins (e.g., H2AX and CHK2) to initiate G1/S cell cycle checkpoint and other cellular responses. Many of these proteins are Mn-dependent or Mn-responsive, including Mre-11, ATM, p53, H2AX, and CHK2.

Studies across numerous neurodegenerative diseases, including AD, have also shown inadequate DNA repair in nuclear and mitochondrial genomes as well as abnormal DDR in both inherited and sporadic neurodegeneration\textsuperscript{84}. AD is characterized by progressive memory loss and cognitive impairment driven by neuronal loss in the hippocampus and cerebral cortex, extracellular neuritic plaques of β-amyloid peptide, and neurofibrillary tangles due to aggregation of hyperphosphorylated tau protein\textsuperscript{85}. Oxidative stress is believed to play a major role in AD and increased DNA damage has been observed in tissue samples from AD patients\textsuperscript{86,87}.
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Base excision repair (BER) is the primary DNA repair pathway for small base modification such as alkylation, deamination, and oxidation. It is believed to play a critical role in development and maintenance of the CNS. A study investigating alteration in BER capacity of AD patient’s brains and found significant BER deficiencies due to limited DNA base damage processing by DNA glycosylases and reduced DNA synthesis capacity by DNA polymerase β. BER impairment was correlated with abundance of neurofibrillary tangles and this suggests that BER dysfunction may play an important role in progression of AD. Another study utilized a cell-free DNA end joining assay to determine if non-homologous end joining (NHEJ) was reduced in nuclear cortical extracts from AD versus control subjects. They observed that end joining activity and protein levels of DNA-dependent protein kinase (DNA-PK) catalytic subunit were significantly lower in AD cortical extracts compared to normal subjects, indicating that repair of DNA DSBs by DNA-PK dependent NHEJ pathway may be deficient in AD. In contrast to the large evidence implicating DNA damage in AD, much less is known about role of DNA damage and repair in the pathogenesis of HD.

DNA response/repair pathways in Huntington’s disease

In HD, increasing CAG repeat length leads to protein structural changes making it more prone to aggregation and suggesting a correlation between repeat length and pathology. DNA repair deficiency in HD was initially reported by studying fibroblasts and lymphocytes of HD patients exposed to ionizing radiation. A study later examined protein and activity levels of major BER enzymes within the striatum (a brain region greatly affected in HD) and cerebellum (a region largely unaffected in HD) in an HD mouse model. Interestingly, both Flap Endonuclease-1 (FEN1) (responsible for 5’-flap endonuclease activity which processes Okazaki fragments) and BER cofactor High mobility group box 1 protein (HMGB1) (involved in long-patch BER) were significantly lower in the striatum compared to cerebellum of HD mice. Furthermore, POLβ was enriched at CAG expansions in the striatum, but not in the cerebellum. This suggests that POLβ strand displacement activity during long-patch BER promotes formation of stable 5’-flap structures at CAG repeats, which are not efficiently removed since FEN1 activity is low in the striatum. Faulty processing of strand breaks by FEN1 contributes to the CAG repeat instability which is observed in HD.

Mismatch and base excision repair are important in the somatic expansion of repeated sequences in mouse models of trinucleotide repeat disorders. DNA mismatch repair is a conserved pathway that maintains genomic stability. Disruptions in mismatch repair have been implicated as a cause of triplet repeat expansions leading to neurodegenerative disease such as HD. Mismatch repair on DNA modulates somatic expansion of repeat tracts. Two complexes are involved in mismatch repair in mammalian cells: MutSα and MutSβ. MutSα contains mutS homolog 2 (MSH2) and mutS homolog 6 (MSH6) which target mismatched bases. MutSβ contains MSH2 and MSH3, which target small insertion-deletions. Knockout of these repair genes was shown to ameliorate HD phenotypes in mice and prevent somatic expansion. HD mouse models of different genetic backgrounds show increased concentrations of MSH3, which is associated with repeat expansion. Furthermore, knockout of MSH2 and MSH3 in cells with 800 CAG•CTG repeats prevented expansion of CAG repeats.

Another DNA repair pathway that has recently been implicated in trinucleotide repeat disorders is the Fanconi anaemia repair pathway. The chromosome 15 locus, which is associated with HD age of onset, contains FAN1, which is a DNA nuclease and candidate for modifying onset of HD by mechanisms similar to topoisomerase-Iβ cleavage or reducing expansion of the CAG repeat. FAN1 is believed to cleave DNA at inter-strand cross-links and is involved in repair with other members of Fanconi anaemia pathway, including FANCD2 and other MMR proteins. FAN1 recognizes branched structures, which mimic DNA repair. This is problematic since repeat sequences (i.e., CAG repeats) form non-helical structures in DNA, such as G-quadruplexes, which FAN1 may target instead of DNA sequences. DNA instability results from large
sections of genomic repeats which leads to aberrant DNA secondary structure formation during replication of DNA repair processes\textsuperscript{105,106}. This suggests that FAN1 may interact with other mismatch repair proteins such as MLH1 to play role in FAN1-driven activity that binds CAG repeats and modulates instability\textsuperscript{92}. Interestingly, FAN1 displays optimal activity in presence of Mn, and if magnesium is used, then the endonucleolytic activity is reduced\textsuperscript{107}. All of these DNA damage and DNA repair pathways have evolved to combat oxidative stress which may be caused by a Mn-handling defect in HD.

**Conclusion and future directions**

There is recent evidence that Mn participates in the pathophysiology of HD by manipulating the activity of enzymes regulating DNA damage response and repair. However, the exact molecular mechanism remain unknown and further examination is necessary. It is unknown whether increased DNA damage in neurodegenerative diseases is a cause rather than a consequence. First and foremost, studies need to be conducted to assess the homeostatic control of Mn in the brain. Understanding how Mn is transported and regulated will offer insights into how Mn plays a role, not only in DNA damage response and repair, but also in HD itself. Many questions also remain about vulnerability of selective neurons in various diseases as well as the contribution of changes due to protein aggregation, oxidative stress, heavy metal toxicity, DNA damage repair, disease onset, progression, and severity. Few studies have examined Mn-levels in different cell types in the brain (e.g., dopaminergic, cortical) to show a similar Mn-deficiency, which is observed in striatal models of HD. Different neuronal lineages and differential time points can be assessed to determine how Mn is playing a role in cellular stress and DNA damage response/repair. Since Mn is a co-factor for many DNA replication and repair enzymes, Mn homeostasis may underlie the phenotypes overserved in HD, and targets of these complex pathways can be assessed to determine what biological response are occurring. Key functional nodes of pathophysiological processes can be manipulated to map functional relationships, identify biological responses, and define targets for interventional strategies. Many of the phenotypes observed in HD may be due to the deficiencies in brain Mn. However, there are still many questions in the field about Mn activation of DNA response pathways, which would help us gain a better understanding of Mn homeostasis as well as DNA damage response and repair in HD.

**References**

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The Molecular and Cellular Architecture of Light-responsive Biological Clocks in the Suprachiasmatic Nucleus

Suil Kim

Abstract

Circadian rhythms are an evolutionarily conserved characteristic of normal physiology. The suprachiasmatic nucleus (SCN) in the hypothalamus is the master pacemaker of autonomous circadian clock of individual cells in the periphery. The SCN sustains circadian rhythms using an autoregulatory transcriptional feedback mechanism and intercellular communications. Light, which serves as a primary synchronizing signal, is transmitted from a subpopulation of retinal ganglion cells to the SCN and induces a shift in the clock phase by regulating transcription and translation of a clock gene. This article concisely reviews the molecular and cellular architecture of the central biological clock in the brain and molecular mechanisms of light-induced clock resetting.

Keywords: circadian rhythm, transcriptional feedback, SCN, neuropeptides

Introduction

Most terrestrial organisms including mammals have evolved to have an internal biological oscillator in most, if not all, of their cells that adjusts the physiology of an organism to cyclic changes in their natural environment\(^1\), including changes in the light-dark cycle, air temperature, food availability, and predator activity. One of the greatest evolutionary advantages for having such an internal biological clock is that organisms can anticipate and prepare for upcoming cyclic environmental changes. Since most environmental changes have diurnal cycles from the influence of the sun, the most ubiquitous biological oscillatory system is based on the daily light-dark cycle that lasts approximately 24 hours, or circadian.

In mammals, the circadian oscillatory system of the whole body is hierarchical. Although not all tissues contribute equally to the circadian system, all tissues do have an internal circadian clock consisting of clock proteins\(^2\). The master pacemaker of the system is located in the suprachiasmatic nucleus (SCN) of the hypothalamus\(^4\), which synchronizes the peripheral circadian rhythm. The SCN, which is located right above the optic chiasm, is one of the first relay regions for retinal ganglion cell inputs\(^5\). As expected from this anatomical organization, the SCN uses light-derived signals as a major synchronizer of its circadian rhythms. This review will discuss the molecular architecture of the autonomous circadian oscillatory unit found universally in all mammalian cells including SCN neurons and the neural circuitry of light-induced resetting of circadian rhythms from the retina to the SCN. Finally, we will review the molecular signaling pathways that underlie light stimulation-induced clock resetting.

The autonomous circadian oscillator model of mammalian cells

Individual cells, including SCN neurons, have a shared molecular circadian oscillatory system comprised largely of three transcription-translation-based autoregulatory feedback loops\(^6\) (Figure 1A). The most essential loop consists of the Brain and Muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-Like 1 (BMAL1), Circadian Locomotor Output Cycles Kaput Protein (CLOCK), Period (PER) 1/2/3, and Cryptochrome (CRY) 1/2 proteins. The genes that encode these proteins are termed "clock genes". The BMAL1 and CLOCK proteins act together as a heterodimeric transcription factor with the basic helix–loop–helix (bHLH)
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and PER-ARNT- single-minded protein (SIM) (PAS) domains. In the daytime, the BMAL1-CLOCK heterodimer activates the transcription of circadian-rhythmic genes (termed clock-controlled genes (CCGs)) as well as that of Per and Cry genes, all of which have an E-box in the promoter region. In the evening PER and CRY proteins accumulate, and early in the night they translocate into the nucleus and bind to BMAL1-CLOCK, causing the formation of a corepressor complex and ultimately leading to transcriptional suppression of the CCGs, Per, and Cry genes. As the auto-repression progresses, PER and CRY protein levels decrease throughout the night as a result of proteasomal degradation. The next morning BMAL1-CLOCK restarts the loop, activating CCG transcription. This cycle of negative-feedback control sets the endogenous period of molecular circadian rhythms.

The second autoregulatory loop induces BMAL1 rhythmic expression. BMAL1 transcription is regulated by two types of transcription factors that bind to Rev-ERβ2 and retinoic acid-related orphan receptor (ROR)-binding elements (RORE): reverse strand of Erythroblastic Leukemia Viral (V-Erb-A) oncogene homolog (REV-ERB) α/β, and RORα/β/γ. RORs activate BMAL1 transcription, BMAL1-CLOCK heterodimer activates transcription of REV-ERBs, and REV-ERBs inhibit ROR-mediated transcription of BMAL1. This loop creates the anti-phase relationship between BMAL1 and PER/CRY expression rhythms.

The third feedback loop is inter-locked with the two other autoregulatory loops (i.e. BMAL1-PER/CRY, ROR-REV-ERB loops). Proline and acidic amino acid-rich basic leucine zipper (PAR-bZip) transcription factors are involved in this loop. A PAR-bZip factor D-box binding protein (DBP) is positively regulated by BMAL1-CLOCK. The DBP competes with Nuclear factor, interleukin-3 regulated (NFIL3), a repressor regulated by the ROR-REV-ERB loop, to activate RORs, a positive regulator of BMAL1.

Since the circadian clockwork is critical for normal physiology, there is functional redundancy of several of the molecular components. Deletion studies have revealed that BMAL1 deletion is the only single gene mutation sufficient to totally disrupt the circadian clockwork and lead to arrhythmic molecular and behavioral phenotypes. Other clock and clock-related gene knockout mice, however, still show circadian rhythms with an aberrant period. Single knockouts of PER1/2/3, CRY1, or RORα result in a shorter period while single knockouts of CRY2 or RORβ result in a longer period. Furthermore, BMAL1 rhythmic expression by the ROR-REV-ERB loop is not required for maintaining the core BMAL1-PER/CRY loop. A number of the clock-controlled genes that have an E-box are also found to have RORE suggesting that they could be regulated by RORs and REV-ERBs as well as BMAL1-CLOCK.

Taken together, the three transcriptional autoregulatory loops are modulated by transcription factors and their repressors that act on the E-box, D-box, and RORE. These inter-locked loops generate autonomous and robust circadian rhythms for target genes, achieving temporal control of physiology.

The retinohypothalamic tract from the retina to the SCN for synchronizing circadian rhythms to the light-dark cycle

In mammals, the eye is the light-receptive organ that transforms light signals into electrochemical signals at the retina. For image-forming functions, light signals are first received by photoreceptors and transmitted to retinal ganglion cells (RGCs) via bipolar cells. For circadian modulations, however, subtypes of RGCs that have melanopsin as a photopigment are able to directly detect light signals. This subpopulation of RGCs, called intrinsically photosensitive RGCs (ipRGCs), occupy about 5% of the total RGC population. There are at least five subtypes (M1-M5) of ipRGCs, differentiated by their varying morphology and electrophysiology. The M1 ipRGCs innervate the SCN: X-gal staining of axonal projections from M1 ipRGCs showed that most of the mouse SCN is densely and bilaterally innervated by M1 ipRGCs (Figure 1B). A more recent study using single M1 ipRGC labeling by alkaline phosphatase staining revealed that the SCN is the only ipRGC-
target region that receives nearly equal amounts of projections bilaterally whereas other areas are contralaterally innervated. Furthermore, the study described a retinotopic map in the SCN where the dorsotemporal and ventromedial regions of the retina send axons to the dorsal and ventral SCN, respectively. This is in contrast to older reports using anterograde tracing with cholera toxin B that the ventrolateral SCN predominantly gets retinal afferents in hamsters and rats. It remains unclear if these discrepancies are due to methodological issues or species-specific characteristics.

The neurocircuitry within the SCN

The SCN is a small hypothalamic region right above the optic chiasm located bilaterally to the third ventricle (Figure 1B). Based on Nissl staining, the SCN is reported to consist of about 20,000 neurons, although Nissl also labels glial cells, that are thought to be exclusively heterogenous GABAergic neurons. Neuropetide characterization using mass spectrometry revealed that there are approximately 190 endogenous peptides expressed in the SCN tissue including vasoactive intestinal peptide (VIP), arginine vasopressin (AVP), and gastrin-releasing peptide (GRP). By percentage of total SCN population AVP+ neurons are the most abundant (37%) followed by VIP+ (24%) and GRP+ (14%). Although there are overlapping expressions of neuropetides in a single neuron, each type is expressed in a certain spatial manner in the SCN (Figure 1B). VIP is mainly localized in the ventral SCN, GRP is located in the central SCN, and AVP is expressed in the dorsal SCN. GRP expression overlaps with that of both VIP and AVP; however, there is no evidence of neurons co-expressing VIP and AVP in the SCN, suggesting that VIP+ and AVP+ neurons are functionally segregated. The SCN also contains universally expressed neuropetides such as neuropeptide S.

The SCN has traditionally been thought to have two functional subdivisions: the core and shell. This hypothesis is mainly based on neuropetide expression patterns, clock gene rhythmicity, and connections to extra-SCN regions. In mice and rats, the core is anatomically located in the ventrolateral SCN and the shell is in the dorsomedial SCN (Figure 1B). The core expresses VIP and GRP, whereas the shell expresses AVP. In addition, the core shows very low or undetectable amplitude in the constant darkness but the shell has rhythmic oscillations, although a more recent paper using bioluminescence imaging suggested that both the core and shell have rhythmic clock gene expression. Light-induced clock gene and c-fos expression, however, is predominantly in the core. The core and shell receive distinct afferents from other brain regions; the output regions, though, are mostly shared and include the subparaventricular zone (SPVZ) and dorsomedial hypothalamus (DMH). The core gets direct photic input from the retina and non-photic inputs from the intergeniculate leaflet (IGL), lateral geniculate nucleus, pretectal nuclei, and raphe nuclei. In contrast, the shell is innervated by the cerebral cortex, basal forebrain, hippocampus, medullary noradrenergic areas, and brainstem cholinergic nuclei.

The connections between different subpopulations of SCN neurons and between the core and shell have been much less studied mainly due to the small size of the SCN and its neurons. Biocytin staining has shown that AVP+ neurons have compact dendritic arborization and their axons mostly terminate inside the SCN. GRP+ neurons form a dense local network within the core but not with the shell. A neuronal tracing study using biotinylated dextran amines injected into the shell showed that the core was not entirely labeled suggesting that chemical synapses between the core and shell might be sparse. Furthermore, another study found that firing patterns from SCN neurons were not cross-correlated, which suggests that fast synaptic transmission mainly by GABA is sparse and weak within the SCN. Rather, non-synaptic volume transmission of neurotransmitters plays an important role in intra-SCN communication. In particular, the role of VIP in the core-shell interaction has been extensively studied since VIP is expressed in retinoreceptive SCN neurons and most SCN neurons express VIP receptor type 2 (VPAC2). VIP- and VIP receptor type 2 (VPAC2)-null mice show arrhythmic behavior and the individual neurons have desynchronized clock-
gene rhythms at the molecular level\textsuperscript{40}, pointing to the necessity of VIP signaling for synchronizing circadian rhythms. Interestingly, VIP can restore molecular circadian rhythms in CRY1/2 double knockout mice that are arrhythmic at the molecular and behavioral levels\textsuperscript{41}, suggesting that VIP might compensate for defects in the autoregulatory feedback loop of clock proteins.

Furthermore, gap junctions play an important role in intercellular communications between interneurons\textsuperscript{42}. Dendro-dendritic and dendro-somatic gap junctions between interneurons are common in the cerebral cortex, although excitatory neurons generally lose gap junctions throughout development\textsuperscript{42}. Indeed, gap junction proteins, including connexin\textsuperscript{46}, help SCN neurons electrically synchronize, which is necessary for normal circadian behavior\textsuperscript{43}. However, it remains unknown how electrical synapses integrate photic information from retinorecipient SCN neurons and give rise to a light-reset endogenous circadian rhythm in the overall SCN and periphery.

The time of the day as a gate for light-evoked resetting of the circadian clock in the SCN

The circadian oscillatory system as a whole allows organisms to predict the environment and optimize behavior via synchronizing signals (e.g. light pulse) from the surroundings. It also maintains a consistent circadian rhythm. A powerful way to study light-induced changes in circadian rhythms is to give the same light stimulus to organisms in constant darkness at different times of the day and examine how their circadian rhythms are affected. It has been well-known that rodents differentially respond to the same light pulse depending on their subjective time of day in constant darkness\textsuperscript{44}. Importantly, since the endogenous period of circadian rhythms is not exactly 24 hours, the subjective time of day can deviate from the actual time of day in the darkness. When a light pulse is given during the subjective daytime there is little or no phase shift in the circadian rhythm, but light given at the subjective night drives phase changes. Light at early night induces phase delays whereas light at late night triggers phase advances. Differential effects of light on resetting the clock phase are shown in vivo at the behavioral level\textsuperscript{44} and ex vivo at the molecular level by application of glutamate receptor agonists\textsuperscript{45,46}, VIP\textsuperscript{47}, and recently optogenetic membrane depolarization\textsuperscript{48}.

Molecular signaling pathways underlying the light-induced resetting of the circadian clock in the SCN

Light inputs for the circadian oscillatory system are transmitted to the SCN via the M1 ipRGCs. Interestingly, conventional rod and cone photoreceptors are not necessary for circadian synchronization\textsuperscript{49} but do serve as complimentary players\textsuperscript{50}. M1 ipRGCs directly respond to mid- and long-wavelength visible light with a photopigment melanopsin and release glutamate and pituitary adenylate cyclase-activating peptide (PACAP) at the synapse of a retinorecipient SCN neuron. Most retinorecipient SCN neurons are in the ventrolateral core region of the SCN and are mainly characterized by, but are not limited, to VIP expression. These neurons also express both glutamate and PACAP receptors.

Retinorecipient SCN neurons express AMPA (\textit{\textalpha}-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (GluA1, GluA2, GluA4), kainate receptors (GluK2, GluK3), NMDA (\textit{\textN}-Methyl-\textit{\textD}-aspartic acid) receptors (GluN1, GluN2C) as well as metabotropic receptors (mGluR1, mGluR5)\textsuperscript{51}. Antagonists of either NMDA or non-NMDA receptors block light-induced resetting of the circadian clock\textsuperscript{42,53}, suggesting that all ionotropic glutamate receptors are necessary for this process. Activation of the ionotropic glutamate receptors triggers membrane depolarization as well as calcium influx directly via NMDA receptors (NMDARs) and indirectly via voltage-gated calcium channels (VGCCs) (Figure 1C). Pharmacological blockade of VGCCs is sufficient to abolish glutamate-induced clock resetting\textsuperscript{54}, suggesting that calcium influx via
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VGCCs is required. However, because NMDAR activation by itself can produce the light-like effect on circadian rhythms\textsuperscript{56}, NMDAR-mediated calcium influx is sufficient for light-induced clock resetting. Taken together, these findings indicate that VGCC activation mainly depends on NMDAR, which is supported by a study in other brain areas\textsuperscript{55}.

PACAP receptors are also expressed in retinorecipient SCN neurons. There are three types of PACAP receptors: PACAP receptor type 1 (PAC1), VIP receptor type 1 (VPAC1), and VIP receptor type 2 (VPAC2)\textsuperscript{56}. They are all Gs-coupled receptors, which activates adenylyl cyclase (AC) and increases intracellular cyclic adenosine monophosphate (cAMP) levels\textsuperscript{56}. PAC1 and VPAC2 but not VPAC1 are expressed in the SCN\textsuperscript{57,58}. PAC1 has a stronger affinity to PACAP than VIP and is widely expressed in the entire SCN\textsuperscript{58}. In contrast, VPAC2 binds equally to VIP and PACAP\textsuperscript{58} and its expression is largely restricted to the ventrolateral core SCN\textsuperscript{58}. PAC1-deficient mice show impairment in light-induced clock resetting in a manner similar to PACAP knockout mice\textsuperscript{59,60}, suggesting that PACAP signaling is mainly mediated by PAC1. Interestingly, PACAP- or PAC1-null mice show relatively normal response to light stimulation at early night, although the late-night response is significantly decreased\textsuperscript{59}, indicating that PACAP signaling is critical for light-induced phase advances at late night.

Both glutamate and PACAP signaling converge onto phosphorylation of cAMP-response element binding protein (CREB) (Figure 1C). Calcium influx mediated by glutamate receptors activates Ca2+/calmodulin-dependent protein kinase II (CaMKII), which phosphorylates CREB. Increased cAMP levels mediated by PACAP receptors activate protein kinase A (PKA), which also targets CREB. Phosphorylated CREBs lead to Per1/2 induction by binding to CREB-responsive element (CRE) on the promoter\textsuperscript{61,62}. A study using CRE decoy and clock gene antisense oligonucleotides\textsuperscript{63} showed that CRE activation is critical for light-induced phase advances in vivo. CRE-mediated Per1 but not Per2 induction was required for glutamate-induced phase advances in vitro. However, it is not yet fully understood how Per1 induction drives phase shifts of the circadian clock.

Transcriptional regulation is not the only clock-resetting mechanism in the SCN: translational control also controls clock-resetting. A light pulse or glutamate application at night induces activation of mitogen-activated protein kinases (MAPKs), including extracellular signal–regulated kinase (ERK)\textsuperscript{64} (Figure 1C). ERK activates MAPK-interacting kinase (MNK), which phosphorylates eukaryotic translational initiation factor 4E (eIF4E). Phosphorylated eIF4E in turn enhances Per1/2 translation\textsuperscript{65}. Blocking eIF4E phosphorylation significantly decreases light-induced clock resetting\textsuperscript{66}, suggesting that eIF4E-mediated regulation of Per1/2 translation is involved in the signaling pathways for light-induced clock resetting. It is still unknown, however, what role translational modulation plays in light induced phase shifts of the circadian clock and how it interacts with transcriptional mechanisms.

Concluding remarks

The molecular circadian oscillatory system is a remarkable evolutionary product which acts as an internal biological clock to facilitate synchronization of physiology to external environment. A heterogenous ensemble of SCN neurons communicate with each other via neurotransmitters and gap junctions to achieve light-induced synchronization of circadian rhythms. At the molecular level, this process is driven by multiple layers of gene regulation, including CREB-mediated transcriptional and eIF4E-mediated translational controls. However, we still do not fully understand a fundamental feature of light-adaptive circadian oscillatory system: the same light pulse differentially resets the clock phase depending on the time of the day. Since circadian clock is based on autoregulatory clock gene transcription, future work will need to focus more on how
the autoregulatory loop is differentially modulated by light at different times of the day. Chromatin modifications may play a role in this process.

Figure 1. Light-adaptive circadian oscillatory system in the SCN.

A. The molecular network of autonomous circadian clockwork largely consists of three autoregulatory feedback loops inter-locked with each other. The core loop (in blue) is formed by BMAL1-CLOCK heterodimer and its repressor PER and CRY. The other two loops are auxiliary. ROR and REV-ERB (in brown) produce rhythmic BMAL1 expression as a positive and a negative regulator, respectively. DBP and its repressor NFIL3 (in green) strengthens the other feedback loops. Triangular and circular arrows indicate transcriptional activation and repression, respectively. B. The neurocircuitry of photic synchronization of circadian rhythms. M1 ipRGCs bilaterally transmit light information in the form of glutamate and PACAP to retinorecipient SCN neurons, which then release VIP to communicate with other SCN neurons and form a synchrony in circadian rhythms. Different color gradients in the left SCN (VIP in red, GRP in green, AVP in blue) indicate the spatial distribution of representative endogenous neuropeptides and peptidergic SCN neurons in the SCN, respectively. The contrast gradient in the right SCN indicates the schematic density of ipRGC innervations. OC: optic chiasm. 3V: third ventricle. C. Molecular signaling cascades underlying the light-induced clock phase resetting. M1 ipRGCs release glutamate and PACAP at the synapse with retinorecipient SCN neurons. Glutamate increases intracellular calcium levels via activation of NMDAR and VGCC, which triggers CAMKII activation. PACAP upregulates cAMP levels via G protein signaling, there-
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by leading to PKA activation. Both active CAMKII and PKA phosphorylate CREB, which enhances Per1/2 transcription. Glutamate also boosts Per1/2 translation though MAPK activation. Increased PER1/2 shifts the clock phase. The diagram at the bottom is the molecular network of the circadian clockwork (see figure 1A).

References

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Feeding the GATOR: Mutations in the amino-acid-sensing GATOR1 complex and the link to focal epilepsies

Lindsay Kozek

Abstract

DEPDC5 mutations have recently been shown to cause focal epilepsies. DEPDC5 is a component of the GATOR1 complex, a complex of three proteins that inhibits mTORC1 signaling in conditions of low nutrient availability. When activated at the lysosomal membrane, mTORC1 inhibits induction of autophagy, the process by which damaged or redundant proteins, protein aggregates, and cell organelles are degraded into their component parts. In loss of function DEPDC5 mutations, this negative regulation is lost and mTORC1 remains at the lysosome, constitutively inhibiting autophagy. Dysregulation of autophagy and hyperactivation of mTOR are both processes implicated in epileptogenesis; however the mechanistic link between DEPDC5 mutations, impaired autophagy, and epileptogenesis has yet to be defined. This review will summarize and discuss the clinical link between GATOR1 mutations and epilepsy, the molecular biology of how the GATOR1 complex regulates mTOR-dependent autophagy, and possible mechanisms linking its loss of function to epileptogenesis.

Key words: epilepsy, autophagy, DEPDC5, NPRL2, NPRL3, GATOR1 complex, mechanistic target of rapamycin

Introduction

GATOR1 (GAP activity toward Bags) is a protein complex that negatively regulates the mechanistic target of rapamycin (mTOR) signaling pathway. Mutations in GATOR1 have recently been identified as a significant cause of familial and sporadic focal epilepsies. mTOR is a ubiquitously expressed serine-threonine kinase that is a master regulator of cell growth, metabolism, and energy homeostasis. mTOR is the catalytic subunit of two different complexes with distinct functions: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Each protein complex contains the same core components as well as several unique accessory proteins. This review will focus on the rapamycin-sensitive mTORC1, which is essential in coordinating anabolic and catabolic processes in response to environmental cues such as stress, energy balance, presence of growth factors, and nutrient availability.

To grow and proliferate, cells must synthesize proteins, lipids, and nucleotides while concurrently inhibiting competing processes such as autophagy. Because biosynthesis, especially of proteins, is a highly energy-demanding process, it is important that it only occur under conditions of sufficient nutrient availability. Components of the mTOR pathway are responsible for sensing the nutrient status of the cell and communicating it to mTORC1, which will promote growth when nutrients, such as amino acids, are available. The mechanism by which cytosolic amino acid levels are communicated to mTORC1 was only recently described in 2013 by Bar Peled and colleagues, who identified two novel protein complexes involved in regulation of mTORC1 signaling: GATOR1 and GATOR2.
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GATOR1 consists of three proteins: disheveled, Egl-10, and pleckstrin domain-containing protein 5 (DEPDC5), nitrogen permease regulator-like-2 (NPRL2), and nitrogen permease regulator-like-3 (NPRL3). The complex is involved in attenuation of mTOR signaling under conditions of amino acid deprivation. GATOR1 attenuates mTORC1 signaling via GTPase-activating protein (GAP) activity toward the Rag guanosine triphosphatases (GTPases). GATOR2 consists of distinct protein components and lies upstream of GATOR1, functioning as a positive regulator of mTORC1.

GATOR1 mutations are now the most frequent known genetic cause of focal epilepsy. Loss of function of GATOR1 leads to mTORC1 hyperactivation and likely impairs mTORC1-dependent autophagy. Impaired autophagy has been implicated in epileptogenesis, suggesting that GATOR1 loss of function mutations may cause epilepsy by preventing proper regulation of autophagy by mTORC1. In this review, I will review the current understanding of the GATOR1 complex and its role in autophagy as well as discuss possible mechanisms linking loss of GATOR1 function to epilepsy.

GATOR1 mutation is a cause of genetic and sporadic epilepsies:

Epilepsy is a condition of recurrent, unprovoked seizures caused by an imbalance of excitatory and inhibitory neuronal input. Epilepsy affects 50 million people worldwide, yet one-third of patients live with uncontrolled seizures because no available treatment is effective. Patients with GATOR1 mutations have been reported to have an especially high rate of drug resistance of up to 78%. Aside from a greatly diminished quality of life, uncontrolled epilepsy also conveys a higher risk of sudden unexpected death in epilepsy (SUDEP).

Mutations in GATOR1 are now known to be a significant cause of familial and sporadic focal epilepsies, which account for about 60% of all epilepsies. The most commonly mutated GATOR1 component is DEPDC5. Mutations in DEPDC5 were initially identified in families with non-lesional autosomal dominant focal epilepsies, specifically in cases of familial focal epilepsy with variable foci (FVEVF), autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), and familial temporal lobe epilepsy (FTLE). Soon after, Scheffer et al. reported both lesional and non-lesional epilepsies in a single family with a germline DEPDC5 mutation. This represents a paradigm shift because genetic epilepsies and genetic malformations have generally been thought of as mutually exclusive. DEPDC5 mutations were subsequently found in focal...
epilepsies, establishing DEPDC5 mutation as one of the most frequent causes of genetic focal epilepsies. To date, GATOR1 mutations have been identified in as many as 5-37% of familial focal epilepsies and up to 1% of sporadic focal epilepsies. DEPDC5 mutations have also been found in a variety of neoplastic processes such as glioblastoma, lung cancer, breast cancer, serous ovarian tumors, and hepatitis C virus-induced hepatocellular carcinoma.

DEPDC5 mutations causing focal epilepsy have a low penetrance with a high phenotypic variability. Some individuals harboring a DEPDC5 mutation are unaffected while affected individuals have variable ages of onset, epilepsy severity, locations of epileptogenic focus, and comorbid conditions such as autism spectrum disorders, intellectual disability, and psychiatric disorders. The explanation for this high variability is unknown but may be due to other genetic, epigenetic, or environmental influences.

Mutations in NPRL2 and NPRL3, the other two components of the GATOR1 complex, were subsequently identified in a variety of lesional and non-lesional focal epilepsies. Importantly, two definite cases of SUDEP were identified in a family with a DEPDC5 mutation and two probable cases of SUDEP were identified in individuals with mutations in NPRL2 or NPRL3. In an analysis of 61 SUDEP cases, 10% (6/61) of cases were found to have DEPDC5 mutations, and DEPDC5 was identified as one of the top 30 SUDEP risk genes in a genome-wide screen. These studies suggest that GATOR1 mutations may confer an increased risk of SUDEP, but more evidence is needed to show a definitive link. An NPRL3 knockout mouse was found to have profound cardiac abnormalities, suggesting a possible mechanism for SUDEP, which may be related to cardiac arrhythmias. As such, GATOR1 mutations may have effects that reach beyond the CNS.

GATOR1 mutations result in pathogenic loss of function

Two-thirds of epilepsy-related DEPDC5 mutations are nonsense or frameshift mutations, and several groups have shown that DEPDC5 transcripts undergo nonsense-mediated mRNA decay, indicating that haploinsufficiency may be deleterious. Individuals with GATOR1 mutation-related epilepsy are heterozygous for the mutation; however, in focal cortical dysplasias, some have postulated that the two-hit hypothesis suggested in tuberous sclerosis may apply. According to this hypothesis, hamartoma formation occurs when patients with one germline TSC1/2 mutation develop a second somatic hit in TSC1/2. Indeed, one sample of resected brain tissue from a patient with a germline DEPDC5 mutation was found to have acquired a second somatic DEPDC5 nonsense mutation in DNA from resected brain tissue but not from blood samples. However, just as not all tumors in tuberous sclerosis show loss of heterozygosity, other samples of brain tissue from patients with DEPDC5 mutations are heterozygous. Another possible mechanism of dysplasia is acquisition of a second hit in another gene involved in regulation of the mTOR pathway, such as PTEN or TSC1/2. For example, individuals in one family with a germline DEPDC5 mutation also inherited mutations in DEPTOR and NF1, both regulators of mTOR, and developed lesional focal epilepsies. Future studies using double knockout animal models will be helpful in testing this hypothesis.

Mutations in the GATOR1 components, which have been identified throughout their entire protein sequences, do not appear to cluster in any specific regions or functional domains. DEDPC5 is a 1603 amino acid protein containing a DEP domain, which is a globular domain found in GTPase-activating proteins, and a DUF3608 domain, and is thought to be important for interaction with NPRL2 and NPRL3. In in vitro co-immunoprecipitation assays, transfected DEPDC5 with a single amino acid deletion mutation in the DUF3608 domain interacted less readily with NPRL2 and NPRL3. However, in the same study, interaction of NPRL2 and NPRL3 with another DEPDC5 variant with an amino acid substitution in the DUF3608 domain was not affected. Additionally, a truncation mutant of DEPDC5 ortholog SEA1/IML1 consisting of amino acids 1-537 did not interact with NPRL2/3, indicating that the DUF3608 domain is not sufficient for
NPRL2/3 interaction\textsuperscript{39}. NPRL2 and NPRL3 both contain a longin domain and a PEST motif. The longin domain, found at the N-terminus, is a domain found in GEF proteins, but GEF activity for NPRL2/3 has not yet been characterized\textsuperscript{12,40}. The PEST motif found near the C-terminus can be found in rapidly degraded proteins. However one study did not detect this type of rapid degradation in NPRL2/3\textsuperscript{12}. The three-dimensional interactions of the GATOR1 complex have not yet been characterized, but the molecular architecture of the orthologous yeast SEA complex has been determined\textsuperscript{39}.

![Diagram of GATOR1 complex proteins](image)

Figure 2. Diagramatic representations of GATOR1 complex proteins\textsuperscript{12,31}.

DEPDC5 contains two identified domains: DUF3608 and a DEP domain. DUF3608 may be important for interaction with NPRL2 and NPRL3, while the DEP domain, for which the protein is named, is found in many proteins with GAP activity. NPRL2 and NPRL3 each contain a longin domain at the N-terminus and a PEST motif at the C-terminus. The functions of these domains in NPRL2/3 are not well characterized.

**GATOR1 function in amino acid sensing and regulation of mTORC1**

The mTOR pathway couples extracellular signals to intracellular metabolic responses. In the presence of nutrients, mTORC1 stimulates cell growth and protein synthesis while inhibiting autophagy and other catabolic processes\textsuperscript{2}. After an organism feeds, serum amino acid levels increase. The Rag GTPases, heterodimers that are tethered to the lysosomal membrane by the Ragulator complex, are converted to their active states upon detection of increased amino acids in the lysosome\textsuperscript{41-43}. The activated Rags are able to bind Raptor, a component of mTORC1, thus recruiting mTORC1 to the lysosomal membrane\textsuperscript{44}. The small GTPase Rheb, when active, is then able to activate mTORC1. mTORC1 negatively regulates autophagy by phosphorylating the autophagy-activating kinase ULK1, inactivating it and preventing induction of autophagy\textsuperscript{45}.
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Withdrawal of amino acids suppresses mTORC1 signaling in both yeast and mammalian cells; additionally, suppressing mTORC1 via starvation or with rapamycin induces autophagy\(^5\). Under amino acid-deprived conditions, GATOR1 prevents the Rag GTPases from recruiting mTORC1 to the lysosome, allowing for autophagy induction \(^9\). In this way, the GATOR1 complex enables the nutrient-deprived cell to break down proteins and organelles in order to generate essential molecular building blocks.

Role of autophagy in the cell

Autophagy is a catabolic pathway by which intracellular proteins and organelles are degraded into their component parts by targeting them to the lysosome for destruction\(^{46}\). The process starts with formation of a phagophore or isolation membrane\(^7\). A double membrane develops around the components to be degraded, forming the autophagosome. A lysosome is recruited and fuses with the autophagosome to create an autolysosome. Components inside the autolysosome are then degraded due to the acidic nature of the lysosomal lumen.

During initiation of autophagy, LC3 is lipated to form LC3-II, which is used as a marker of autophagy induction and autophagosomes\(^7\). Autophagy can be generalized or targeted to specific organelles and is implicated in a variety of processes such as embryonic development, the adaptive response to starvation, and axonal homeostasis\(^{14}\). The process is essential for clearance of protein aggregates, defective proteins, and dysfunctional organelles. If autophagy is dysregulated, old organelles will accumulate within cells and cause oxidative stress\(^{46}\). Defects in autophagy have been implicated in many neurologic disease processes, including several pediatric neurodegenerative and neurometabolic diseases, adult neurodegenerative diseases such as Alzheimer’s disease and Huntington disease, autism spectrum disorders, cortical malformations, and epilepsies, suggesting that tight regulation of this process is essential for proper nervous system development and function\(^{6,47,48}\).

Effects of GATOR1 loss in in vivo and in vitro models

Loss of GATOR1 function prevents attenuation of mTORC1 during amino acid starvation, leading to constitutive inhibition of autophagy\(^8\). In cells with shRNA-mediated DEPDC5 knock-down, mTORC1 localizes constitutively to the lysosomal surface regardless of amino acid availability\(^9\). Furthermore, overexpression of DEPDC5 was enough to block amino acid-induced translocation of mTOR to lysosomal surface\(^9\).

*Drosophila* with defective GATOR1 components show increased cell growth and sensitivity to nutrient starvation\(^{49}\). *NPRL2/3* mutants fail to activate autophagy in response to low levels of amino acids but can be rescued by overexpressing DEPDC5\(^{49}\). *DEPDC5/-* rat embryonic fibroblasts also fail to regulate cell growth appropriately in response to amino acid starvation\(^{50}\). Moreover, in characterizing GATOR1 mutations in Drosophila, Wei and colleagues\(^{51}\) found that no homozygous *DEPDC5* mutants were born, suggesting that complete loss of *DEPDC5* is embryonic lethal. Loss of *DEPDC5* was embryonic lethal in rats and caused global growth delay\(^{50}\).

Linking mTOR, autophagy, and epileptogenesis

Much remains unknown about the link between dysregulated mTOR signaling, impaired autophagy, and epileptogenesis. Hyperactivation of mTORC1 is believed to play a significant role in the epileptogenic process\(^{52-54}\). Several mechanisms have been proposed, although most have been in the context of tuberous sclerosis (TS). For example, some cortical specimens from TS patients show a decreased number of inhibitory GABAergic interneurons and decreased levels of glutamate decarboxylase, an enzyme essential for GABA synthesis, suggesting that mTOR hyperactivation may alter the balance of excitatory and inhibitory input, favoring hyperexcitability\(^{15}\).
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Hyperactivation of mTORC1 during development leads to defects in cellular maturation, increased size of neuronal cell bodies and dendritic arbors, abnormalities in myelination, and defects in circuit formation\(^55,56\). \textit{DEPDC5-/-} rats show abnormal cortical development and died as embryos while heterozygous rats exhibit cytomegalic dysmorphic neurons and aberrantly positioned cells suggestive of abnormal neuronal migration\(^50\). It is possible that these abnormalities in cortical development result in focal cortical dysplasias and lead to seizures.

Autophagy in the brain plays a critical role in neuronal processes including axonal homeostasis, prevention of accumulation of protein aggregates, and mitochondrial homeostasis\(^57\). Impaired autophagy in neurons after disinhibition of mTORC1 has been shown to contribute to epileptogenesis in TSC1 and PTEN knock-out models\(^14\). McMahon and colleagues\(^58\) studied epileptogenesis and autophagy using a conditional knock-out of \textit{Atg7}, an essential autophagy gene. \textit{Atg7} knock-out mice developed spontaneous seizures and had decreased survival\(^14\). The mice did not show mTORC1 hyperactivation as determined by phospho-S6 level demonstrating that impaired autophagy alone is sufficient to promote epileptogenesis.

Conclusions

In the last several years, the GATOR1 protein complex has been identified and characterized as a modulator of mTORC1 in response to amino acid availability. Mutations in its three components, DEPDC5, NPRL2, and NPRL3, have been identified as an important cause of sporadic and genetic focal epilepsies. Studies to elucidate the pathogenic mechanism have demonstrated that loss of GATOR1 results in mTORC1 hyperactivation and impaired autophagy.

The link between GATOR1 loss of function, hyperactivated mTOR signaling, impaired autophagy, and epileptogenesis has yet to be well defined and remains an open area of research. Future studies should also aim to explain the phenotypic variability of epilepsies with GATOR1 mutations and determine the cause of associated focal cortical dysplasias.

Identification of novel drug targets offers new options for patients with drug-resistant epilepsies and further characterization of how GATOR1 causes epileptogenesis may provide new treatment options for a particularly drug-resistant population.

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The insula in nicotine, alcohol, and amphetamine addiction

Joe Luchsinger

Abstract

Alcohol and substance abuse are chronic diseases with significant morbidity and mortality. The personal and economic devastation that stems from these illnesses is immense, reaching well beyond the afflicted individual. The majority of those addicted experience relapse while trying to maintain abstinence. Substantial resources have been allocated toward elucidating the underlying pathophysiology of substance abuse, yet the neuronal mechanisms underlying addiction remain largely unresolved. Addiction has been modeled in three phases: “binge/intoxication”, “withdrawal/negative affect”, and “preoccupation/anticipation”. Due to the role of the insula in interoception and integrating internal state with external stimuli, the insula likely plays a different role in each phase. This paper will review the insular circuitry related to addiction followed by an assessment of the evidence for the role of the insula in addiction to various drugs of abuse in both animal and human models.

Function of the insula

The insula is a large and complicated region of the lateral mammalian brain, sometimes referred to as the fifth lobe. It is known to be involved in a vast number of behavioral and cognitive processes, many of which are beyond the scope of this review. In brief, the insula is hypothesized to integrate internal and external stimuli. One fundamental role of the insula is interoception, which is the awareness of internal body states, specifically of imbalances in body states. An example of this is the presence or absence of thirst, the driving force that keeps the body within a specific hydration range. Thirst is correlated with middle insular fMRI response to the oral administration of water. Changes in internal stimuli can drive or alter a wide variety of behaviors. As such, many studies are working to identify the role of the insula in specific sensations and behaviors. For example, the administration of lidocaine, which inactivates the insula, has elucidated the role of the insula in visceral sensation. Lidocaine, by preventing action potential propagation via the inhibition of sodium channels, ameliorates the normal malaise induced by lithium exposure in rodents. Further, the insula shows increased activity during wine-tastings, a complex task of integrating taste and olfactory stimuli, in sommeliers when compared to controls. Insular activation has also been demonstrated in homeostatic processes such as temperature sensation, heartbeat awareness, gut and bladder distention, dyspnea, and sexual arousal.

Though there is debate over the comparability of food overconsumption and addiction (largely due to the requirement of food for survival), hunger, like drugs of abuse, can produce a very strong appetitive drive. Some individuals process food stimuli differently than the rest of the population and are prone to excessive food intake, and it is thought that this same circuitry may be co-opted by drugs of abuse. The insula is a key node in a broad range of processes related to immediate food intake and the effect of that intake on the body. This information coupled with the extensive conditioned taste aversion (CTA) literature identifies the insula as an important waypoint in ingestive learning and memory.

Emotional and physical discomfort is often characteristic of the withdrawal/negative affect stage of addiction. Evidence suggests that insular processing of these internal stimuli may be another link between the insula
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and the neuronal correlates of addiction. Both stress and anxiety increase during acute and chronic abstinence from drugs of abuse and are believed to be critical factors in relapse\textsuperscript{34,35}; animal studies modeling withdrawal have provided evidence of dysregulation of the hypothalamic-pituitary-adrenal axis\textsuperscript{36} and, consistent with this, have shown that levels of the stress-related neuropeptide corticotropin-releasing factor (CRF) increases in the extended amygdala\textsuperscript{36–38}. These changes contribute to a negative emotional state. The insula sends excitatory projections onto CRF neurons in the extended amygdala, providing direct insular access into this canonical stress-related region. A meta-analysis by Phan et al.\textsuperscript{39} found that stress and anxiety contribute significantly to the malaise experienced during abstinence. Anxious individuals have incongruous interoception and tend to overestimate their physiologic response to stressors and are more likely to associate stressors with perceived anxiety-related internal stimuli\textsuperscript{40,41}. When faced with abstinence, these individuals are at risk for effectively over-perceiving their negative physical symptoms.

It has been hypothesized for some time that the conscious perception of physical discomfort can be altered and exacerbated by emotional states\textsuperscript{42,43}. Described as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage”\textsuperscript{34}, pain provides information about potentially negative deviations from homeostasis. One of the early clues about the role of the insula in pain perception is from a study conducted in 1988 of patients that experienced unilateral strokes of various sizes, all of which encompassed the insula\textsuperscript{35}. In these patients, strokes induced asymbolia for pain such that patients could feel pain but did not exhibit an appropriate emotional or escape response. Four years later, it was discovered that a tumor impinging on the posterior aspect of a patient’s right insula reversibly inhibited contralateral pain perception\textsuperscript{44}. Furthermore, in a study in which stereotactically-placed depth electrodes were used to stimulate different parts of the insula in patients with intractable epilepsy it was found that pain was intermittently elicited through stimulation of the contralateral dorsal posterior insula. It should be noted that other non-painful stimuli were also elicited upon stimulation of the anterior and posterior insula, indicating a role for overlapping regions of the insula in a variety of sensory experiences\textsuperscript{45,46}. Like primary sensory cortices, pain sensation appears to be somatotopically organized in the insula, and the subjective pain network includes the posterior insula\textsuperscript{47,48}. Although still a point of contention, the dorsal posterior insula may respond specifically to pain\textsuperscript{49–51}. Recently, Orenius et al.\textsuperscript{52} showed that there is a positive correlation between pain and emotions on insular BOLD signal. Positive and negative emotional states can alter sensory perception and processing through a broad neural network of cortical and subcortical regions\textsuperscript{53–55}. Both emotional and visceral sensations are handled by the anterior insula, and negative emotional contexts can change how visceral stimuli are interpreted\textsuperscript{55}. A human fMRI study demonstrated anxiety-dependent changes in hippocampal and insular BOLD response to painful stimuli\textsuperscript{56}, consistent with the putative role of the insula in emotional processing\textsuperscript{39}.

Stress and anxiety are important emotional characteristics that can alter and enhance addiction behavior\textsuperscript{57,58}. Reciprocal connections with the amygdala, a region key in regulating emotional responses to fear and pain stimuli, further supports the notion that the insula is important for responding to stressors. Stress elevates measurements of blood flow to the insula and increases both the likelihood of initiating drug taking as well as drug reinstatement during abstinence\textsuperscript{36,59–62}. The negative affect experienced during the withdrawal phase has been hypothesized to increase the propensity for reinstatement, and insular activity has been shown to correlate with anticipation of aversive events\textsuperscript{63,64}. Patients with posttraumatic stress disorder (PTSD), typified by a recurrent and intrusive intensely stressful state, show hyperreactivity of brain regions associated with emotional regulation, including the insula, as well as decreased insular expression of the inhibitory neurotransmitter gamma-aminobutyric acid\textsuperscript{65–67}. PTSD also correlates with an overall decrease in insular grey matter density and volume\textsuperscript{68,69}. In situ hybridization and autoradiographic studies demonstrate insular expression of the receptor for CRF\textsuperscript{70}. Although animal studies that inhibit the CRF system or its outputs have demonstrated some efficacy in preventing behavioral sensitization\textsuperscript{71–76}, human studies have yet to prove...
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efficacious. Despite unsuccessful initial trials, the converging roles of the insula have made it a favorable candidate in addiction research.

Architecture of the insula

The German psychiatrist Johann Christian Reil first described the region now known as the insula (“island” in Latin) in 1796. More than half a century later, in 1858, the region was dubbed the island of Reil in the first edition of Anatomy: Descriptive and Surgical, a publication that would go on to become the renowned Gray’s Anatomy. Compared to non-human primates the human insula is structurally distinct though not significantly larger overall than predicted by our size. The insula is located within the Sylvian fissure, deep to the temporal and frontal lobes, and has since been divided into 3 diagonally-oriented sub regions based on cytoarchitecture: agranular, dysgranular, and granular. The most posterior of these three regions is the granular insula, which is characterized by the broad granular layers II and IV. The dysgranular insula is a strip located just anterior to the granular insula and has thinner and less dense granular layers II and IV than the granular insula. The most anterior region is the agranular insula which lacks granular cells altogether. The insula can also be divided into anterior and posterior divisions. The anterior division encompasses the entire agranular region as well as some of the granular and dysgranular regions. The posterior division includes parts of both the dysgranular and granular regions. Information is generally processed in a posterior to anterior direction, traveling from granular insula to agranular insula and increasing in complexity of integration throughout this progression. The insula has a broad array of connections to both cortical and subcortical structures which differ anteriorly and posteriorly (Figure 1). For a comprehensive overview see one of several reviews on this subject. Anteriorly, afferents that project to the gustatory insula from the ventral posteriomedial thalamus were identified in primates. The anterior cingulate also sends projections to the anterior insula. Primate tracing studies by Friedman et al. in the 1980s demonstrated afferent projections to the posterior insula from the amygdala and the secondary somatosensory cortex, while others have demonstrated afferents from an array of other sensory areas. Various dorsal thalamic nuclei relay spinal information to the insula. In humans, connectivity between the insula and the amygdala and somatosensory cortices has been shown using probabilistic tractography algorithms. Afferents from structures like the parabrachial nucleus (PBN), ventral tegmental area (VTA), locus coeruleus (LC), dorsal raphe (DR), and substantia nigra pars compacta also allow the insula to integrate information directly from the brainstem.

The insular efferents to structures classically involved in addiction circuitry generally arise from the anterior insula. One of the densest outputs from the insula is the amygdala. These projections innervate multiple amygdalar nuclei and are particularly strong in the lateral, basolateral, and central nucleus of the amygdala. Regions of the extended amygdala, such as the bed nucleus of the stria terminalis (BNST), also receive insular input. Insular projections to the nucleus accumbens, in addition to cortical regions that relay information to the hippocampus, have also been identified using anterograde tracing studies. Connections to the hippocampus have been reviewed by Augustine. In brief, the dysgranular insula sends projections to the perirhinal cortex while the agranular insula predominantly sends projections to the entorhinal cortex. Recently, functional connectivity between the insula and hippocampus and the insula and amygdala was demonstrated in humans using depth electrodes and cortical stimulation in epileptic patients. In addition, the insula sends significant information to the prefrontal cortex, and tracing studies have revealed that the insula also has dense internal interconnections.

The insula takes in multimodal information about the body and incorporates that information, continually increasing in complexity as the insula transitions from posterior to anterior. For example, actual magnitude
of thermal stimuli was correlated with posterior insula activity, while perceived magnitude was correlated with anterior\textsuperscript{134}. Though this was a small study, it illustrates the posterior-to-anterior trend toward conscious perception. Information is then distributed widely throughout the brain to areas previously demonstrated to be involved in different stages of addiction. This positions the insula at a potentially critical node in the neurocircuitry of substance abuse.

![Diagram of insular structure with key efferents and afferents.](image)

Figure 1: Overview of insular structure with key efferents and afferents.

**Nicotine and the insula**

According to the World Health Organization (WHO), the annual worldwide death toll of smoking is 6 million people and predicted to top an aggregated 1 billion people during the 21st century\textsuperscript{102}. International efforts to stymie the increasing social and economic costs are underway. Genome-wide association studies (GWAS) identified a single nucleotide polymorphism (SNP) in the nicotinic acetylcholine receptor alpha-5 subunit that correlated with an increased likelihood of developing nicotine addiction\textsuperscript{104,105}. This polymorphism was shown to decrease acetylcholine receptor function, likely leading to decreased neuronal activity following acetylcholine release. Subsequently, an fMRI study examining the correlation between the presence of this SNP and brain activity after exposure to smoking-related cues found that, counterintuitively, although these individuals are more likely to experience nicotine dependence, they show reduced BOLD signals across multiple brain areas, including the insula\textsuperscript{105}. While preclinical studies generally show that decreasing insular activity can reduce drug seeking behaviors, the divergent finding in the acetylcholine system underlines the complexity and sometimes differing roles of various neurotransmitters in addiction and the importance of investigating each of these systems.

However, Kuhn et al.\textsuperscript{106} had mixed findings with respect to insular response to nicotine. They found a correlation between insular activity and self-reported craving on fMRI, yet related cues did not increase insular blood flow. This contrasts with a publication by Engelmann et al.\textsuperscript{107} that demonstrated that nicotine cues increased insular (posterior) activation. Cerebral response to nicotine cues, gathered prior to smoking cessation, has also been shown to be predictive of abstinence maintenance in participants who previously smoked but had undergone treatment successfully\textsuperscript{108}. This study additionally demonstrated lower functional connectivity between the insula and areas involved in cognitive control in those who failed to maintain abstinence, which they posit may indicate decreased cognitive control\textsuperscript{108}. 

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In the time since a 2007 retrospective study\(^{109}\) reported decreased cigarette craving after insular damage the insula has experienced a renaissance in the addiction field. This study compared 19 patients with damage to the insula to 50 patients with damage elsewhere in the brain; both populations were smokers (greater than one pack a day on average) at the time of injury. The group investigated two outcomes: the continuation or cessation of smoking and the relative ease with which smoking was halted. Though there was not a significant difference in the overall percentage of patients that stopped smoking, for those that did quit smoking insular damage, as opposed to lesions elsewhere, was correlated with being able to quit smoking easily, indicating a disruption in the drive to smoke.

Following this retrospective study, a prospective study compared the effect of insular vs. non-insular strokes on smoking cessation\(^{110}\). Of the 110 total participants, 27 had strokes involving the insula. At one year out 70.4% of the patients with insular strokes had quit smoking compared to only 30.1% of the patients with non-insular strokes. Insular stroke patients also rated the experience of quitting smoking as less challenging\(^{110}\). A later study demonstrated an additive effect of strokes with damage to the basal ganglia in addition to the insula on smoking cessation\(^{111}\).

Animal studies have been used to elucidate a causal relationship between insula manipulation and nicotine addiction. Work by Pushparaj et al.\(^{112}\) evaluated the effects of posterior insula electrical stimulation on nicotine self-administration in rats. Rats were tested under fixed and progressive ratios of reinforcement and, in both cases, electrical stimulation decreased nicotine-seeking behavior. Additionally, posterior insula stimulation decreased nicotine-seeking after priming with either nicotine injection or nicotine-cue exposure in previously extinguished rats. Electrophysiology showed that electrical stimulation inhibited insula neuron activity\(^{112}\). These findings were consistent with a previous study by the same group in which pharmacological inhibition of the insula through the combined administration of the \(\gamma\)-aminobutyric acid agonists muscimol and baclofen by intracranial injection decreased nicotine seeking\(^{113}\). Hollander et al.\(^{114}\) discovered decreased self-administration of nicotine after global inhibition of the insula using the hypocretin-1 receptor antagonist SB-334867. Importantly, these findings appeared to be relatively specific to nicotine as they were not generalizable to food consumption\(^{112-114}\).

Alcohol and the insula

According to a 2015 NSDUH study of the US population over 15 million individuals over the age of 18 have an alcohol use disorder\(^{115}\). Correlational human imaging studies have assessed the connection between alcohol exposure and insular activity. One study compared the response of ten alcoholics to visual alcohol-related stimuli to those of ten controls by first providing them with sips of alcohol prior to entering the fMRI scanner and measuring changes in cerebral blood flow after showing them cues of alcohol or non-alcohol related beverages\(^{116}\). Alcoholics but not non-alcoholic controls had a robust increase in bilateral insular activity when shown an alcohol-related cue\(^{116}\). In a larger study of 326 people classified as heavy drinkers (5 or more drinks per occasion for men and 4 for women, at least 5 times in the last month), individuals were presented with alcohol and non-alcohol related cues during the fMRI scan. The insula was activated by alcohol-related cues, and this was strongly correlated with the degree of the alcohol use disorder in the participant\(^{117}\).

The ritual of drinking alcohol is more than a visual sensory experience, but rather is multi-sensory, involving vision, audition, touch, taste, and olfaction. The feel of the bottle, the sound of the ice and liquid hitting the glass, the aromatic compounds released, and the taste when imbibed all play a role in the experience of drinking. To investigate the brain regions involved in craving, Olbrich et al.\(^{118}\) exploited the various sensory modalities involved in preparing a glass of alcohol. Before entering the positron emission tomography scanner, alcoholic patients were queried for their favorite alcoholic beverage. Once in the scanner, a bottle of their favorite alcohol was brought into the room on a tray, held in front of their faces, a glass was poured, and finally,
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a sponge was used to absorb the beverage and hold it in front of their nose for olfaction. Participants then rated their level of craving. As a control, the same procedure was performed with a bottle of mineral water. Alcohol exposure but not water increased the craving response experienced by the participants. Cerebral blood flow also increased in the insula after alcohol exposure\textsuperscript{18}. Unfortunately, the study did not distinguish between anterior and posterior insula. However, logical arguments could be made for both regions. Due to the multi-sensory nature of the study, it could be postulated that the posterior insula should be activated. However, the conscious perception of the multi-sensory information could point to anterior insular activity. It is likely that both are true, but future research will be needed to delineate the anterior posterior division of labor in cue base activation.

The search for genetic links to alcoholism has been an area of heavy investment. One of the candidate genes related to alcoholism is tachykinin receptor 1 (TACR1), a G\textsubscript{qi} G protein-coupled receptor that responds to substance P. Inhibition of TACR1 has been shown to lower symptoms experienced by detoxified alcoholic patients\textsuperscript{19}. TACR1 polymorphisms were later demonstrated to predict BOLD signal in the insula as well as symptoms reported in the Study of Addictions: Genetics and Environment Genome Wide Association study (SAGE GWAS)\textsuperscript{20,121}.

The predilection for overconsumption of alcohol despite negative social and economic consequences makes alcoholism particularly difficult to treat\textsuperscript{3}. With the advent of tools like optogenetics, nuanced circuit analysis can be performed in awake behaving animals. Seif et al.\textsuperscript{122} performed a particularly elegant optogenetic experiment regarding insular efferent and alcohol abuse. By employing a rodent model of self-administered ethanol intake, the group avoided the issue of lacking contingency (volitional exposure). After the animals were trained to self-administer alcohol, they were divided into two groups: one that continued receiving normal ethanol and another that had ethanol mixed with the aversive tastant quinine, allowing them to explore alcohol consumption that was resistant to negative consequences. The group then used optogenetics to inhibit specific projections from the insula and the prefrontal cortex to the nucleus accumbens. In both cases, light-driven inhibition of these projections decreased intake in the quinine + alcohol group while leaving the alcohol-only group unchanged. These findings did not generalize when sucrose was substituted for alcohol or when shock was substituted for quinine. They went on to show that these results were NMDA-dependent. These findings indicate a specific possible role for these circuits in the resistance of alcohol consumption to negative consequences. Coupled with the human imaging studies, there appears to be increased insular activity in appetitive behavior and craving toward alcohol.

Amphetamine and the insula

Amphetamine is a potent central nervous system stimulant and can be used as a drug of abuse. It works at the synapse by blocking breakdown, inhibiting uptake, and increasing release of monoamines. The insula has dense expression of D1 receptors and receives significant dopaminergic innervation, measured by immunohistochemical tyrosine hydroxylase expression, that increases from posterior to anterior\textsuperscript{123,124}. This could be one avenue by which drugs of abuse affect the insula.

A small study by Riccardi et al.\textsuperscript{125} investigated sex differences in dopamine release after amphetamine exposure using positron emission tomography. Six females and seven males were given orally-administered amphetamine, completed the Sensation Seeking Scale-Form V, and entered the scanner\textsuperscript{126}. Insular dopamine was negatively correlated with sensation seeking in men but not women, signifying potential sex differences in how amphetamine alters dopamine release and cognition in the insula. Though limited by sample size, this study illustrates the value of future examination of potential sex differences in the effects of amphetamine and other substances of abuse.
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Immunohistochemical analysis has shown that µ-opioid receptors are also highly expressed in the insula\textsuperscript{127}. Using the positron emission tomography ligand [(11)C]carfentanil, a synthetic opiate that binds to opioid receptors, Colisanti et al.\textsuperscript{128} showed that high dose amphetamine administration given three hours before entering the scanner reduced radiolabeled carfentanil binding in the insula. Because opioids already present in the synapse prevent carfentanil from binding, the slower carfentanil binding shown in this study indicates increased endogenous opioid release after amphetamine administration.

Contreras et al.\textsuperscript{131} have performed multiple preclinical experiments to investigate the role of the insula in amphetamine response. In this 2007 study, rats underwent conditioned place preference training (CPP) to amphetamine in a paradigm in which amphetamine was delivered in the less preferable of two connected chambers. This procedure changed the rat’s preference such that the initially less preferred chamber became the preferred chamber after amphetamine pairing. Injection of lidocaine, known to inhibit cortical transmission, into the insula reversibly eliminated this preference when given prior to the session; this effect was specific to the insula, and the amphetamine paired place preference returned after lidocaine inhibition wore off. In a later publication, the group used the protein synthesis inhibitor, anisomycin, to prevent insular CCP memory consolidation\textsuperscript{131}. Once again, rats acquired a place preference to amphetamine. Half of the rats were then re-exposed to the chamber for place preference testing while the other half was not, after which both groups received intracranial injection of anisomycin into the anterior insula. Only the group that re-experienced the chambers eliminated their amphetamine place preference, a result that lasted for weeks after exposure\textsuperscript{131}. Posterior insular injections had similar but less robust effects. Thus, it appears that intervention for amphetamine addiction may be more potent in anterior regions that correlate with increased complexity of representations. However, the specific circuitry involved has yet to be elucidated.

Summary

Anatomical studies show insular connectivity with a broad array of somatosensory-, motivational-, and addiction-related brain regions. Human studies have predominantly been correlational with respect to the role of the insula in addiction related tasks, though some recent interventional works show promise\textsuperscript{132,133}. Preclinical studies, which are increasing in frequency, are beginning to dissect the causal implications of insular activity on substance abuse animal models. Studies using intracranial injections to modify protein expression or neural activity and optogenetic studies are beginning to provide a far more nuanced understanding of the circuitry involved in different substances of abuse\textsuperscript{6,122,131}. Work by Seif et al.\textsuperscript{122} illustrates the type of future projects that will help to differentiate cell types and projection specific correlates of insular-related addiction behavior. Further dissection of the specific role of the insula in each phase of addiction will open new avenues for intervention. Currently it appears the insula plays related but distinct roles in each of these phases. During intoxication, the insula allows for the conscious recognition of the immediate sensory effects of the drug (e.g., the taste of ethanol or the cardiovascular effects of cocaine). Initial multi-sensory information is processed in the posterior insula, and signals are then relayed to the anterior insula for higher order integration and conscious perception. In the negative affect phase, it is also possible that the physiologic effects of drug withdrawal are made consciously aware via this same information flow through the insula. In preoccupation, communicated environmental cues passed from other brain regions (e.g., the prefrontal cortex) cause reactivation of the interoceptive representation of the drug leading to conscious urges for the drug. In general, information is processed in a posterior-to-anterior direction from granular to dysgranular to agranular cortex, though there is significant crosstalk across all regions of the insula. While the distinct differences of the anterior and posterior insula await further confirmation, the agranular cortex of the anterior insula appears to be the most potent potential therapeutic target. As this work progresses, it may illuminate novel targets for therapeutic intervention.
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Protein interactions in the presynaptic active zone mediate synaptic vesicle docking, voltage-gated calcium channel recruitment to facilitate short-term presynaptic plasticity

Lauryn N. Luderman

Synaptic vesicle exocytosis is tightly regulated at the presynaptic active zone to ensure precise and reliable neurotransmission. The active zone is an electron-dense structure located adjacent to the presynaptic plasma membrane of synapses in both vertebrates and invertebrates. The evolutionarily conserved molecular structure of the active zone is made up of a large protein complex that is responsible for organizing synaptic vesicles near fusion machinery and Ca\textsuperscript{2+} sources. Importantly, active zone proteins are sensitive to intracellular signaling molecules and mediate the presynaptic response following repeated high frequency stimulation. This review describes the structure and function of individual protein families that contribute to the principal functions of the active zone, and will address the need for a viable vertebrate model to examine potential roles for active zone proteins in the pathophysiology of developmental disease.

Keywords: presynaptic active zone, synaptic vesicle, short-term presynaptic plasticity.

Introduction

Synapses are specialized cell junctions through which information is passed from a presynaptic neuron to a postsynaptic cell, usually another neuron. Arrival of an action potential to the presynaptic terminal depolarizes the plasma membrane, allowing voltage-gated Ca\textsuperscript{2+} channels to open and Ca\textsuperscript{2+} to flow into the presynaptic terminal. The influx of Ca\textsuperscript{2+} triggers the fusion of synaptic vesicles to the plasma membrane on the presynaptic terminal and the exocytosis of synaptic vesicles pools that have been packaged with neurotransmitters from the presynaptic terminal into the synaptic cleft. The neurotransmitters can then bind to receptors on the postsynaptic neuron.

Synaptic vesicle exocytosis occurs specifically at an electron-dense region adjacent to the presynaptic plasma membrane known as the presynaptic active zone (AZ). AZs are found in a variety of synapses, including large neuromuscular junctions, specialized ribbon synapses in photoreceptor cells, and both excitatory and inhibitory central synapses\textsuperscript{1}. Evolutionarily conserved AZ proteins have homologous structure and function in both vertebrates and invertebrates\textsuperscript{2}. Interactions between AZ proteins, synaptic vesicle proteins, and exocytosis machinery are essential for fast, efficient synaptic neurotransmission.

In this review, I will discuss the structures and the major roles of the core presynaptic AZ proteins (Figure 1). I will also discuss how these individual molecular components work together to perform the principal functions of the AZ, including priming synaptic vesicles for fusion, recruiting voltage-gated Ca\textsuperscript{2+} channels to the presynaptic membrane, and facilitating short-term presynaptic plasticity. Although significant progress has been made in determining how individual AZ proteins contribute to its function after synaptogenesis, the role of AZ proteins during neurodevelopment and how AZ protein defects may lead to developmental disease pathology remains unknown and requires more exploration.
Molecular components of the AZ

*Rab3-interacting molecules (RIMs)*

Rab proteins are small GTPases that cycle between active GTP-bound and inactive GDP-bound states to regulate membrane vesicle trafficking in all cells. In neuronal cells, interactions between Rab3 and effector proteins are essential for transport, tethering, and docking of synaptic vesicles to presynaptic terminals. A family of Rab3-interacting molecules (RIMs), identified through yeast two-hybrid screening, was found to localize to the AZ and facilitate synaptic vesicle fusion. A single RIM gene has been identified in *C. elegans*, whereas vertebrates express 4 RIM genes (*RIMS1-4*) that encode six isoforms (RIM1α, RIM2α, 2β, 2γ, RIM3γ, and RIM4γ). RIM isoforms contain a combination of five identified domains: a N-terminal zinc finger domain surrounded by α-helices, a central PDZ domain, two C-terminal C2 domains (C2A and C2B), and a proline-rich sequence found between the C2 domains. Only RIM1α and RIM2α contain the complete set of RIM domains. Interestingly, the C2 domains of RIMs do not have binding sequences for Ca²⁺, a factor that is different from another Rab3 effector protein, rabphilin.

RIM proteins bind to other synaptic and AZ proteins, suggesting RIM functions as an essential scaffolding protein in the AZ. The α-helices in the N-terminal region of RIM proteins bind to Rab3 in a GTP-dependent manner while the adjacent zinc finger domain binds to Munc13-1, another AZ protein discussed below. The formation of a RIM/Munc13/Rab3 tripartite complex is necessary for the tethering and priming of synaptic vesicles for subsequent vesicle fusion and neurotransmitter release. Furthermore, perturbation of the RIM1/Munc13 interaction reduces the readily releasable pool of synaptic vesicles and the recruitment of Munc13 to the AZ.

The central PDZ domain of RIM proteins binds to ERC proteins, another presynaptic AZ protein that is later discussed, and to N- and P/Q-type Ca²⁺ channels. Interaction between RIM-PDZ domain and voltage-gated Ca²⁺ channels is essential for the recruitment of these channels to the AZ. Knockout of all RIM isoforms in mice decreased voltage-gated Ca²⁺ channel localization at the AZ, altered Ca²⁺ influx into the presynaptic terminal and decreased neurotransmitter release. However, expression of a RIM-PDZ domain containing fragment in these RIM knockout neurons rescued all RIM-deficient phenotypes, including voltage-gated Ca²⁺ channel localization.

Additionally, interactions between RIM-binding proteins (RIM-BPs) and the proline-rich sequence of RIM is implicated in voltage-gated Ca²⁺ channel localization to the AZ and is further discussed below. Much less is known about the functional interactions of RIM C2 domains: some studies suggest the C2A domains bind to SNARE proteins and the C2B domains bind to α-liprin, synaptotagmin-1, and Ca²⁺ channels, but these results were not confirmed in studies assessing the C2A and C2B domain crystal structures. Expression of RIM2γ, which contains only a RIM-C2B domain, in HEK293 cells modulates voltage-gated Ca²⁺ channel opening in vitro but does not rescue the RIM knockout phenotype in cultured neurons. Interestingly, RIM3γ and RIM4γ, two short RIM-C2B only isoforms, not only modulate voltage-gated Ca²⁺ channel inactivation but also inhibit anchoring of synaptic vesicles near channels in the AZ. This suggests RIM isoforms play different roles in modulating voltage-gated Ca²⁺ channels. Additional studies are needed to determine how the opposite functions of RIM1α and RIM3/4γ on voltage-gated Ca²⁺ channel localization modulate short-term presynaptic plasticity.
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**Figure 1.** The molecular components of the presynaptic active zone (AZ). **Left:** Diagram of a synapse. Synaptic vesicles fill the presynaptic terminal and are targeted to the AZ (red) for Ca\(^{2+}\)-dependent exocytosis. The postsynaptic terminal contains neurotransmitter receptors embedded in plasma membrane adjacent to the postsynaptic density (dark gray). **Right:** Schematic of the core molecular components of the AZ. RIM, Munc13, RIM-BP, Liprin-a, ERC and Piccolo/Bassoon work together to mediate synaptic vesicle docking and voltage-gated Ca\(^{2+}\) channel recruitment to the AZ to facilitate short-term presynaptic plasticity.

**Munc13s**

Munc13 proteins are a highly conserved, large, multi-domain protein family that localizes to the presynaptic AZ of vertebrates and invertebrates. The role of Munc13 proteins in the AZ was first identified in *C. elegans* when an “uncoordinated” phenotype and loss of synaptic vesicle release was observed in *unc-13* mutants, a homolog of mammalian Munc13\(^{18}\). Five Munc13 genes have been identified in mammals; however only Munc13-1, -2, and -3 are expressed in the brain\(^{19}\). The Munc13-2 gene is alternatively spliced to produce a brain specific protein isoform (bMunc13-2) and a ubiquitously expressed isoform (ubMunc13-2)\(^{20}\). All Munc13 proteins share homologous central and C-terminal domains, including a C1 domain, that bind to the second messenger diacylglycerol (DAG), a Ca\(^{2+}\)-dependent C2B domain, a Munc homology domain (MUN), and a Ca\(^{2+}\)-independent C2C domain located at the C-terminus\(^{19,21}\). In contrast, the N-terminal region is divergent among Munc13 proteins: Munc13-1 and ubMunc13-2 contain an additional Ca\(^{2+}\)-independent C2A domain and a calmodulin (CaM) binding site whereas the N-terminal of bMunc13-2 and Munc13-3 do not contain an identifiable domain\(^{19,22}\). The C2A domain of Munc13 isoforms homodimerize, thereby regulating Munc13 function\(^{23}\). Interaction of Munc13 with RIM proteins disrupts these Munc13 C2A homodimers and is necessary for synaptic vesicle priming and fusion\(^7\). The structure and localization of Munc13 proteins suggest an involvement in DAG- and Ca\(^{2+}\)-mediated synaptic vesicle release at the AZ\(^{24}\).

In vivo evidence supporting a role for Munc13 in mediating synaptic vesicle priming includes knockouts of Munc13/UNC-13 in mouse hippocampal neurons and *C. elegans*, respectively, in which synaptic vesicle fusion and neurotransmission was abolished in both vertebrates and invertebrates\(^{18,25,26}\). Prior to exocytosis, interaction between a partial *transi*-SNARE complex and syntaxin-1 holds a synaptic vesicle close to the
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plasma membrane thereby “priming” the vesicle for release\textsuperscript{27}. However, syntaxin-1/\textit{trans}\textendash SNARE complex formation is regulated by the Sec1/Munc18-like (SM) protein Munc18-1, which locks syntaxin-1 in a ‘closed’ conformation. In vitro studies determined the Munc13-1 MUN domain mediates the transition of syntaxin-1 from a ‘closed’ conformation in a complex with Munc18 to an ‘open’ conformation that is part of the \textit{trans}\textendash SNARE complex\textsuperscript{28}. A recent study identified specific residues in the linker region of syntaxin-1 that, upon interaction with the Munc13-1 MUN domain, induces a conformational change that allows syntaxin-1 to form the SNARE complex\textsuperscript{29}. Additionally, the Munc13 CaM recognition motif, C1, and C2B domains regulate synaptic vesicle priming are implicated in facilitating short-term presynaptic plasticity and will be discussed further below\textsuperscript{22, 30}. These results suggest that Munc13 proteins prime synaptic vesicles for fusion and during repeated action potentials can potentiate synaptic vesicle release in a Ca\textsuperscript{2+}-dependent manner.

\textbf{RIM-BPs (RIM-binding proteins)}

RIM-BPs were discovered through yeast two-hybrid screens and GST pull-down assays designed to identify new potential binding partners for RIMs\textsuperscript{6}. Three RIM-BP genes have been identified in vertebrates (\textit{RIM-BPI} -3) whereas a single gene has been identified in \textit{Drosophila} (\textit{drbp})\textsuperscript{6, 31}. All RIM-BP isoforms contain three SH3 domains, one centrally positioned and two located at the C-terminus, and 2-3 fibronectin III domains\textsuperscript{32}. RIM-BP SH3 domains bind to proline-rich sequences of RIMs and α-subunits of voltage-gated Ca\textsuperscript{2+} channels\textsuperscript{33}.

Voltage-gated Ca\textsuperscript{2+} channel anchoring and AZ organization are two primary functions of RIM-BPs in the AZ. Drbp hypomorphs disrupt not only Ca\textsuperscript{2+} channel localization, but also the molecular organization of the \textit{Drosophila} AZ suggesting that RIM-BPs functions as a channel anchor and a scaffolding protein for other AZ molecular components\textsuperscript{33}. The role of RIM-BPs as an AZ scaffold in \textit{Drosophila} is also important for preventing premature, ectopic AZs on axon terminals\textsuperscript{34}. However, RIM-BPs acting as an essential AZ scaffolding protein is not supported in a mammalian model. Conditional knockout of RIM-BP1/2 in mice did not significantly alter the ultrastructure of hippocampal neurons but did result in altered synaptic transmission kinetics\textsuperscript{35}. This study suggests that while RIM-BP does not function in direct synaptic vesicle exocytosis, this AZ component is necessary for reliable neurotransmitter release. Further studies identified RIM-BP2 as the major RIM-BP paralog in excitatory hippocampal neurons in mice, and loss of RIM-BP2 affects P/Q-type Ca\textsuperscript{2+} channel localization, ultimately resulting in altered short-term synaptic plasticity\textsuperscript{36}. Additional evidence supports a role for RIM-BP in modulating synaptic plasticity by affecting the maintenance of the readily releasable pool of synaptic vesicles in \textit{Drosophila}\textsuperscript{37}. In summary, voltage-gated Ca\textsuperscript{2+} channel anchoring is a conserved function of RIM-BPs in both vertebrates and invertebrates which ultimately can impact synaptic plasticity.

\textbf{Liprin-α proteins}

Liprin-α is a molecular component of the AZ but is also found in cells outside of the nervous system. For example, Liprin-α was found to complex with other proteins to drive migration of cancer cells\textsuperscript{38}. In the brain, the liprin-α protein family is involved in the maturation of synapses. A single liprin-α gene is expressed in invertebrates: \textit{syd-2} in \textit{C. elegans} and \textit{diliprin} in \textit{Drosophila}. Vertebrates express four liprin-α genes (liprin-α1, -α2, α3, and -α4)\textsuperscript{39}. Structurally, liprin-α is composed of an N-terminal coiled-coil region that contains liprin homology domains LH1 and LH2, and three C-terminal SAM (sterile-α-motif) domains\textsuperscript{40}. The N-terminal LH domains can homodimerize or form heterodimers with other presynaptic AZ proteins such as the RIM and the ERC family of proteins\textsuperscript{13, 41}, C-terminal SAM domains of liprin-α proteins interact with LAR-type receptor phosphotyrosine phosphatases\textsuperscript{42}, CaMKII\textsuperscript{43}, and CASK\textsuperscript{44}, a MAGUK protein localized both
pre- and postsynaptically. Additionally, liprin-α interacts with GRIP1, an AMPA receptor binding protein, suggesting liprin-α acts as a scaffolding protein in the postsynaptic terminal.\(^{45}\)

While liprin-α proteins perform multiple functions outside of the nervous system, this review will focus on liprin-α function in the presynaptic AZ. Initial studies examined liprin-α function at the AZ in syd-2 C. elegans mutants. A syd-2 loss of function mutation resulted in a larger AZ and disruption of synaptic vesicle accumulation.\(^{46}\) Interestingly, a gain of function mutation in the syd-2 L1H1 domain can promote synapse assembly in the absence of SYD-1, a C. elegans specific protein required for synapse formation.\(^{47}\) The gain of function syd-2 mutation suppressed the syd-1 mutant phenotype, which includes uncoordinated movement and defects in egg-laying behavior through enhanced homodimerization and binding to ELKS.\(^{48}\) Results from C. elegans syd-2 mutants suggest liprin-α is critical for proper presynaptic assembly. Moreover, liprin-α has been shown to be involved in synaptic neurotransmission and AZ molecular dynamics. Acute knockdown of liprin-α2 following shRNA transfection of cultured rat hippocampal neurons resulted in a decrease in the total recycling pool of synaptic vesicles, including the readily releasable pool, and a decrease in synaptic vesicle release probability.\(^{49}\) Furthermore, liprin-α2 deficient neurons exhibited decreased expression of known liprin-α binding partners, RIM1 and CASK, and a fluorescence recovery after photobleaching (FRAP) assay showed a reduction in recovery of RIM and CASK to the AZ in liprin-α depleted synapses.\(^{49}\) Results from invertebrate and in vitro vertebrate studies suggest liprin-α proteins function in assembling the presynaptic AZ by interacting with other AZ proteins, and play a role in mediating synaptic transmission by influencing synaptic vesicle accumulation and the dynamics of vesicle release machinery such as RIMs and CASK.

**ERC family of proteins**

Unlike other AZ proteins, the function of ERC proteins at the AZ is less defined and difficult to interpret. The ERC gene was originally discovered as ELKS, a novel gene that activates RET tyrosine kinase in papillary thyroid carcinomas.\(^{50}\) ERC was later rediscovered as a Rab6-interacting protein (Rab6IP2)\(^{51}\), and as a core AZ component renamed CAST\(^{52}\) (or ERC, an acronym of ELKS, Rab6IP2, and CAST)\(^{10}\). Vertebrate species contain two ERC paralogous genes, ERC1 and ERC2, whereas C. elegans and Drosophila contain a single homologous gene known as ELKS and bruchpilot, respectively.\(^{53, 54}\) ERC in vertebrates has alternative splice sites, and the resulting isoforms are differentially expressed: shorter ERC isoforms are primarily expressed in neuronal cells whereas longer isoforms are found in nonneuronal tissues.\(^{10}\) ERC proteins are predicted to be composed of four coiled-coil domains and contain both a PDZ-binding consensus sequence and a Rab-binding domain at its C-terminus.\(^{55}\) While the protein structure suggests ERC proteins function as scaffolding proteins at the AZ, vertebrate and invertebrate studies differ with respect to defining the role of ERC proteins in AZ assembly and mediating neurotransmitter release.

In contrast with other model organisms, the ERC homolog Bruchpilot (BRP) appears to play a significant role in AZ assembly and neurotransmitter release in Drosophila. RNAi knockdown of BRP disrupts the ultrastructure of synaptic AZs and neurotransmitter release in larval motor neurons and adult photoreceptors.\(^{54}\) Additional studies show that loss of BRP also alters Ca\(^{2+}\) channel localization to Drosophila AZs.\(^{56}\) However, examination of the ERC homolog ELKS in C. elegans suggests functional redundancy with RIM in the AZ.\(^{53}\) RIM and ELKS interact in the AZ via the RIM-PDZ domain, but loss of ELKS does not affect RIM localization or function in synaptic vesicle release. Further, knocking out ELKS in C. elegans did not alter synaptic transmission or animal behavior, a phenotype that is observed in RIM knockout experiments. Similarly, ERC proteins may play a redundant role with RIMs in the assembly of the vertebrate AZ: complete disassembly of the AZ was observed in a quadruple knockout of presynaptic RIM and ERC proteins.\(^{57}\) ERC proteins in the vertebrate AZ also appear to mediate neurotransmitter release. Deletion of ERC2 in mice caused a large
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large increase in neurotransmitter release and in the pool of readily releasable vesicles in inhibitory hippocampal neurons; however, knocking out ERC2 did not alter the ultrastructure of the synapse. Although, the role of ERC in assembly of the AZ may be synapse specific: the ribbon synapse and AZ size in rod photoreceptors are reduced in ERC2 knockout mice. In contrast with results from ERC2 knockout, double knockout of both ERC proteins in mice led to a 50% reduction in neurotransmitter release and a reduction in the release probability of synaptic vesicles in inhibitory hippocampal neurons. Additionally, loss of ERC proteins from inhibitory synapses caused a significant reduction in action potential-triggered Ca\(^{2+}\) influx into the presynaptic terminal without affecting Ca\(^{2+}\) channel localization to the AZ. A recent study found that double knockout of ERC in mice also resulted in reduced neurotransmission of excitatory hippocampal neurons but no defects in Ca\(^{2+}\) influx. These data together suggest that ERC proteins may perform different functions in sub-types of synapses, and its role in mediating synaptic vesicle release may have important implications in the pathophysiology of neurologic diseases.

Bassoon/Piccolo

Bassoon and Piccolo are two large proteins that were identified in a screen for structural components of rat brain synaptic junctions. Although originally thought to be vertebrate specific AZ proteins, Fife and Bruch-pilot in *Drosophila* are structurally related homologs to Piccolo and Bassoon and perform similar functions in the invertebrate AZ. Piccolo and Bassoon have similar amino acid sequences and contain ten highly conserved regions termed Piccolo-Bassoon homology domains that include zinc finger motifs and coiled-coil structures. Additionally, Piccolo contains a C-terminal PDZ domain and two C2 domains, one of which undergoes a conformational change upon binding to Ca\(^{2+}\). The structures of and the interactions of Piccolo and Bassoon with other AZ proteins suggest that these proteins function as scaffolding proteins in the AZ.

Components of the AZ are trafficked from the trans-Golgi network to the presynaptic terminal on precursor vesicles. Piccolo, Bassoon, and ERC exit the Golgi on the same precursor vesicle (Piccolo-Bassoon-ELKS/CAST transport vesicle, PVT) and traffic to the synapse via microtubules and associated motor proteins. Knockdown of Piccolo and Bassoon in cultured neurons led to an accumulation of ERC2 on Golgi membranes, suggesting that Piccolo and Bassoon are required for ERC2 trafficking to the AZ.

In addition to mediating trafficking of ERC proteins to the AZ, Bassoon and Piccolo may play a role in regulating synaptic vesicle release. Double knockdown of Bassoon and Piccolo in mice did not affect neurotransmission in either excitatory or inhibitory synapses but did affect presynaptic vesicle clustering. However, Bassoon or Piccolo loss of function mutations in mice did not affect AZ ultrastructure of central synapses, likely due to functional redundancy with other AZ proteins. In contrast, Bassoon and Piccolo are implicated in the assembly and maturation of ribbon synapses at retinal photoreceptor cells. Interaction of Bassoon with RIBEYE, a ribbon synapse protein, is critical for the formation and function of the ribbon synapse as loss of Bassoon-RIBEYE interaction prevents association of the ribbon to the photoreceptor AZ and greatly reduces synaptic transmission. Additionally, knockdown of the main Piccolo isoform, Piccolino, disrupts the maturation and ultrastructure of ribbon synapses in mouse photoreceptor cells. These results suggest that, in addition to trafficking AZ components, Bassoon and Piccolo also act as scaffolding proteins in both central and sensory synapses by organizing synaptic vesicles and vesicle release machinery.

Presynaptic AZ function

*Synaptic vesicle priming*

One of the main functions of the presynaptic AZ is to prime synaptic vesicles for exocytosis by docking vesicles close to the plasma membrane and fusion machinery, thereby creating a readily releasable pool (RRP) of vesicles. Neurotransmitter release following the arrival of an action potential to the presynaptic terminal is
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dependent on the size of the RRP and vesicle release probability ($P_{vr}$). As discussed previously, AZ molecular components RIM, Munc13, and Rab3 form a tripartite complex that is essential for priming vesicles for release and maintenance of the RRP; genetic manipulation of these components or complex formation significantly impairs neurotransmitter release and size of RRP. However, despite electrophysiological evidence of alterations in synaptic transmission in knockout animals, the ultrastructural defects of vesicle docking are not always observed. For example, RIM but not Munc13 mutants show a reduction in RRP size that parallels a loss of vesicle docking in glutaraldehyde-fixed tissue. The inconsistency in the phenotype in these studies was resolved using an optimized fixation technique for examining vesicle docking. A recent study used a combination of high-pressure freezing electron tomography (HPF-EM) and 3D reconstruction of AZs on hippocampal slices from Munc13 knockout mice to reveal that Munc13 is required for docking of synaptic vesicles to the plasma membrane and those vesicle docking defects significantly alter the size of the RRP.

Although the Munc13-RIM interaction is the central component for mediating synaptic vesicle priming and maintaining the RRP, other AZ proteins are suggested to contribute to this presynaptic AZ function. As discussed above, determining specific ERC protein function in the AZ has been difficult, but evidence suggests ERC proteins regulate synaptic vesicle priming machinery. Double knockout of ERC proteins from excitatory hippocampal neurons resulted in a decrease in RRP size and neurotransmitter release without affecting the $P_{vr}$. ERC function in regulating the RRP appears to be specific to excitatory synapses as no changes to the RRP were observed in ERC deficient inhibitory hippocampal neurons. Interestingly, a quadruple knockout of RIM/ERC isoforms from mouse hippocampal neurons completely disrupted AZ organization and synaptic vesicle docking, but the RRP was only mildly affected compared to ERC or RIM knockout alone. Using HPF-EM, this study found fusion competent vesicles located in the interior of the pre synaptic terminals that can still undergo spontaneous exocytosis and transverse to the membrane following hypertonic sucrose stimulation. This result suggests other mechanisms may be responsible for vesicle translocation and fusion in the absence of an AZ. Furthermore, ERC1 is responsible for recruiting bMunc13-2, a brain specific Munc13-2 isoform, to the AZ of a subset of hippocampal synapses. ERC1 binds to a coiled-coil domain in the bMunc13-2 N-terminal, and loss of this interaction results in defective synaptic vesicle priming. The ERC1-bMunc13-2 interaction appears to act in a manner analogous to that of the Munc13-RIM interaction controlling synaptic vesicle priming in a small subset of hippocampal neurons, but further studies are needed to determine how this interaction might contribute to synaptic plasticity of these microcircuits.

Recruitment of voltage-gated Ca^{2+} channels

Localization of voltage-gated Ca^{2+} channels to the AZ facilitates Ca^{2+}-dependent synaptic vesicle exocytosis by placing vesicle fusion machinery and a Ca^{2+} source within close proximity. The interactions between RIMs and RIM-BPs are important for the recruitment of N- and P/Q-type Ca^{2+} channels to the AZ. Interestingly, the size of the AZ of hippocampal glutamatergic terminals is proportional to the number of Ca^{2+} channels recruited to the AZ by RIM. Additionally, an increase in AZ size scales with an increase in RRP and $P_{vr}$, suggesting that function and morphology of AZ are linked.

The ERC family of proteins may also be involved in the recruitment of voltage-gated Ca^{2+} channels to the AZ. Bruchpilot, an ERC homolog, is required for voltage-gated Ca^{2+} channel clustering at the Drosophila AZ, but whether vertebrate ERC proteins function in a similar manner is in question. Double ERC knockout in mouse inhibitory hippocampal neurons reduces Ca^{2+} influx into the presynaptic terminal following stimulation but does not alter Ca^{2+} channel localization. This result suggests that ERC proteins modulate Ca^{2+} channel opening either directly or via recruitment of another modulatory protein to channel. However, ERC1 knockouts are embryonic lethal, and so it is necessary to use a conditional knockout approach to remove
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ERC protein expression only after completion of synaptogenesis. Therefore, voltage-gated Ca\textsuperscript{2+}-channels may be retained in the presynaptic membrane following removal of ERC proteins, but trafficking of voltage-gated Ca\textsuperscript{2+}-channels to the AZ during development was not examined. Other studies have determined that ERC proteins directly interact with the $\beta_3$ subunit of voltage-gated Ca\textsuperscript{2+}-channels using co-immunoprecipitation and fluorescent co-localization imaging techniques. Co-expression of ERC2 and voltage-gated Ca\textsuperscript{2+}-channels in BHK cells shifts the voltage dependent activation of the channels towards a hyperpolarized state. These results suggest that ERC proteins bind to and modulate physiological properties of voltage-gated Ca\textsuperscript{2+}-channels, but more work is necessary to address whether ERC proteins are needed to traffic Ca\textsuperscript{2+}-channels to the AZ during vertebrate development.

Short-term presynaptic plasticity

Synaptic plasticity refers to an adjustment in synaptic strength and signal transmission efficiency that occurs in response to repeated stimulation. Synapses can be strengthened or weakened both pre- and postsynaptically. While long-term synaptic plasticity can last hours, short-term plasticity lasts on a scale of milliseconds to minutes. Short-term presynaptic plasticity can be separated into three classes: facilitation, depression, and augmentation. Synapses will exhibit presynaptic facilitation when arrival of a second stimuli within milliseconds of the first evokes a larger presynaptic response than the first. An opposite effect occurs during short-term presynaptic depression: a second stimuli evokes a smaller response. Augmentation is response enhancement following sustained presynaptic activation and lasts longer than presynaptic depression or facilitation. Short-term presynaptic plasticity has many functional roles such as mediating stimulation and directional selectivity in sensory cells.

Short-term presynaptic plasticity is influenced by Ca\textsuperscript{2+} influx and the pool of releasable synaptic vesicles. Munc13 is a key AZ component involved in mediating short-term presynaptic plasticity. Munc13-1 and ub-Munc13-2 both contain an evolutionarily conserved CaM recognition motif and bind to CaM in a Ca\textsuperscript{2+}-dependent manner during synaptic activity. Overexpression of wild-type Munc13 proteins or CaM-insensitive Munc13 mutants in Munc13-1/ubMunc13-2 double knockout hippocampal neurons reveals that the Munc13-CaM interaction increases synaptic vesicle priming and modulates refilling of the readily releasable pool of vesicles at the AZ.

The Munc13-C1 domain is a target for DAG second messenger signaling. Despite not affecting synaptic vesicle priming or evoked release, mutation of the Munc13-1 C1 domain to prevent DAG binding in mice led to a decrease in the overall RRP size in hippocampal neurons, suggesting a higher $P_{vr}$ in Munc13-1 C1 mutant synapses. Following repeated high-frequency stimulation, high $P_{vr}$ and depleted RRP stores resulted in presynaptic depression in mutant mice. This study suggests that Munc13 proteins function in maintaining a RRP following repeated high frequency stimulation and that this process is mediated by DAG second messenger signaling.

The C2B domain of Munc13 proteins also contributes to short-term synaptic plasticity by binding to both Ca\textsuperscript{2+} and phospholipids. Disruption of Ca\textsuperscript{2+}-binding to the C2B domain in neuronal cultures impaired synaptic vesicle release induced by repeated action potentials; however, a mutation that enhances Ca\textsuperscript{2+}-dependent phospholipid binding to the Munc13 C2B domain increased neurotransmission in response to both single and repeated action potentials, suggesting that increased Munc13 binding to the plasma membrane in response to Ca\textsuperscript{2+}-binding contributes to short-term presynaptic plasticity. Crystal structure analyses of a Munc13-1 fragment containing its C1, C2B, and MUN domains revealed that the DAG- and Ca\textsuperscript{2+}-binding sites of the C1 and C2B domain are located in close proximity to each other, and mutations that disrupt the interface between these Munc13 domain impair synaptic vesicle release, priming, and short-term plasticity.
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Collectively, these results suggest that Munc13 proteins respond to presynaptic terminal accumulations of Ca^{2+} and DAG second messengers following periods of repeated high stimulation by increasing association with the plasma membrane, which in turn increases synaptic vesicle priming to help maintain a RRP to contribute to short-term presynaptic plasticity.

**Conclusions**

The active zone is a highly-conserved presynaptic structure in both vertebrates and invertebrates. Genetic, molecular, structural, and electrophysiological studies have revealed the role of each major AZ protein family in organizing the structure of the AZ and how they contribute to the overall AZ function of mediating fast, efficient neurotransmission.

In addition, the AZ may play a significant role in development. Numerous genetic and clinical studies have implicated disruption in synapse development and function as a key part of the pathophysiology of different developmental, neurological, and psychiatric diseases. Recently, mutations in AZ proteins have been found in patients diagnosed with autism among other developmental disorders. However, studying the impact of AZ protein mutations on development is difficult to accomplish in a mouse model. For example, constitutive knockout of ERC1 is embryonic lethal in mice, which forces researchers to resort to using conditional knockout strategies and precludes examination of ERC1 function prior to synaptogenesis.

Using zebrafish as a vertebrate model to determine the role of AZ proteins during development circumvents many of the issues that plague mouse developmental models. Zebrafish exhibit rapid development that can be easily monitored from a single-cell stage to freely monitored larvae. Additionally, application of genetic editing and transgenic techniques in combination with live, in vivo imaging in zebrafish would allow researchers to determine how a defective AZ protein may contribute to the pathophysiology of neurodevelopmental diseases such as autism.

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The role of parvalbumin-expressing interneurons and endocannabinoid signaling in addiction-related pathology

Kevin M. Manz

Abstract

Illicit drug use is a pervasive socioeconomic issue with complex neurobiological effects. While emphasis has been placed on changes in glutamate- and dopaminergic signaling in the nucleus accumbens (NAc), little is known how inhibitory neurotransmission, particularly via parvalbumin (PV)-expressing interneurons (INs), contributes to drug-related behavioral adaptations. Recent evidence suggests that PVINs may critically regulate circuit activity in the NAc despite making up only 0.7-1\% of its neurons. PVINs are fast-spiking, electrically-coupled inhibitory neurons that uniquely express the cannabinoid receptor type-1 (CB1R) on a fraction of synaptic terminals. Although CB1R-dependent plasticity mechanisms have become increasingly investigated at glutamatergic synapses in the NAc, no studies have looked at how endocannabinoid regulation of PV-INs modulates motivational behavior. Understanding the dynamics of this microcircuit may have therapeutic implications for any condition in which maladaptive motivational behavior is a diagnostic feature, including addiction, depression, and schizophrenia.

Keywords: Accumbens, addiction, endocannabinoids, GABA, interneurons, parvalbumin, plasticity

Introduction

The prevalence of illicit drug use in the United States continues to rise, with 9.4\% of people 12-years-of-age or older reporting illicit drug use in the last month. It is estimated that 10\% of people meet DSM-V criteria for substance use disorder at some point in their lives, imposing an annual societal cost of $484 billion. While substance abuse continues to receive greater attention from healthcare providers, mechanisms by which drugs of abuse trigger cellular and molecular changes in the brain remain elusive. The nucleus accumbens (NAc), a neuroanatomic region in the ventral forebrain, is critically involved in transforming complex motivational states into goal-director behavior. It is thought that drug-induced synaptic adaptations in the NAc lead to maladaptive behavioral states characteristic of addiction-related pathology.

Early studies of drug-induced synaptic events emphasized changes in dopamine (DA) output from the ventral tegmental area (VTA), a mesencephalic structure rich in DA-containing perikarya. Drugs of abuse collectively enhance DA-dependent neuromodulatory processes in mesoaccumbens fibers projecting to the NAc, indicating that phasic DA release (500 Hz) from these neurons contribute to the rewarding properties of drugs of abuse. DA-induced synaptic scaling of glutamatergic synapses in the NAc prompted investigators to examine ways in which excitatory signaling in the NAc mediated reward learning. The importance of glutamatergic signaling in the NAc is demonstrated by studies in which blockade of N-methyl-D-aspartate (NMDAR) and amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPAR)-type glutamate receptors attenuates drug-induced place conditioning, indicating that excitatory input via AMPAR- and NMDAR in the NAc shapes maladaptive motivational learning to drugs of abuse.

While drug-induced changes in DA and glutamatergic signaling continue to be the focus of examination, the role of γ-aminobutyric acid (GABA)-mediated inhibitory neurotransmission in the NAc remains unexplored. GABA is the principal inhibitory neurotransmitter in the mammalian central nervous system (CNS) and the primary ligand expressed by interneurons in ventral (NAc) and dorsal striatal networks.
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GABAergic interneurons, particularly parvalbumin-expressing interneurons (PV-INs), have been proposed as cellular nodes through which neuronal output from the NAc gates goal-directed motivational behavior. PV-INs in the NAc are unique in that they are the only intrinsic cell-type to express the cannabinoid 1 receptor (CB1R), the cotype receptor for endogenous cannabinoids (eCBs) in the CNS. The eCB system has been implicated in various drug-related behavioral states, including cue-induced reinstatement of reward seeking and intensification of drug craving during abstinence. For this reason, GABAergic signaling via PV-INs will be reviewed in the context of (a) NAc-specific circuit function and dynamics, (b) eCB-dependent plasticity mechanisms, and (c) their role in addiction-related behavior.

Examining the neurocircuitry and principal output neurons of the NAc

The NAc is a ventral striatal structure at the limbic-motor interface. It receives afferent glutamatergic input from (a) the basolateral amygdala (BLA), encoding emotionally salient environmental stimuli, (b) the ventral subiculum of the hippocampus (vHipp), relaying contextually-relevant information, and (c) the medial prefrontal cortex (mPFC), relaying information related to top-down executive processing. Monosynaptic excitatory input from these regions project onto GABAergic medium spiny neurons (MSNs) that comprise 90-95% of the cell types in the NAc. MSNs are quiescent, medium-sized neurons with bistable up- and down-state resting membrane potentials at -60 and -80 mV, respectively. Distinct populations of MSNs in the NAc can be distinguished based on whether they express dopamine D1 (D1R) or dopamine D2 (D2R) receptors, with D1R-expressing MSNs constituting the direct pathway and D2R-expressing MSNs the indirect pathway. D1R MSNs project reciprocally to midbrain DA areas including the VTA and substantia nigra pars compacta (SN), whereas D2R MSNs project first to the ventral pallidum (VP) before innervating the VTA and dorsomedial nuclei of the thalamus (Figure 1).

Direct-indirect terminology is inherited from dichotomous D1R and D2R MSN pathways in the dorsal striatum (dStr). While D1R and D2R MSNs represent distinguishable populations of MSNs in the NAc, 45-50% of D1R MSNs collateralize onto neurons in the VP, indicating that D1R and D2R MSNs may have overlapping projection targets. Optogenetic studies utilizing photoactivatable channelrhodopsin (ChR2) indicate that D1R and D2R MSNs promote and oppose reward-related behavior, respectively. It has been hypothesized that drugs of abuse attain their addictive potential by enhancing excitatory drive onto D1R-expressing MSNs. This is supported by receptor bindings assays showing that tonic DA release in drug-naïve mice binds preferentially to D2R and that drug exposure raises extracellular DA sufficiently to occupy D1R. D1R activation has been shown to increase AMPAR expression on D1R MSNs via protein kinase A (PKA)-dependent phosphorylation, indicating that phasic DA release may augment excitatory postsynaptic strength at D1R MSNs.

Diverse interneuron populations in the NAc

The remaining 5-10% of cells in the NAc are GABAergic and cholinergic interneurons with differentiable morphological, physiological, and molecular expression profiles. A subset of GABAergic interneurons stain triple-positive for somatostatin, neuropeptide-Y and nitric oxide synthase and exhibit low-threshold spiking activity upon intracellular current injection. Cholinergic INs (CINs) are choline acetyltransferase-positive neurons that exhibit prolonged spike afterhyperpolarization potentials, preventing high-frequency spike trains from being elicited at CIN-to-MSN synapses. Unlike PV-INs, CINs express D1R and D2R (65-80%) highly, indicating mesolimbic DA release may influence cholinergic output from these neurons. PV-INs, the interneuron population focused on hereafter, are fast-spiking, GABAergic neurons that stain strongly positive for PV, GABA, and the rate-limiting GABA synthetic enzyme, glutamate decarboxylase-67 (GAD67). Each IN subtype targets distinct subcellular domains of MSNs that may spatiotemporally regulate input-
output properties of MSNs. Thus, diverse IN populations in the NAc may work collaboratively to control NAc-dependent motivational behavior.

**Inhibitory PV⁺ interneurons in NAc and striatal microcircuits**

In the NAc, 63% of fast-spiking interneurons are PV⁺ and 74% express CB₁R, indicating that most PV-INS in the NAc express CB₁R at presynaptic terminals. While the physiological relevance of PV is unclear, PV-expressing cells in other tissues exhibit Ca²⁺-chelating properties due to EF hand motifs at N- and C-termini of the PV protein. PV-INS are aspiny with dense varicosities along minimally branching dendritic arbors. Axonal fields are diffuse with numerous branch points emerging from moderately sized somata (16-18 μm), highlighting the large diameter within which a single PV-INS can influence neighboring cellular activity. Varicosities at distal dendritic branch points represent electrical dendro-dendritic synapses between adjacent PV-INS connected via connexon gap junctions. Unitary current injection into a PV-INS results in a time-locked change in membrane potential in adjacent PV-INS, indicating that PV-INS are electrically coupled to one another. Electrical coupling between nearby PV-INS may sync and entrain PV-INS activity to carefully coordinate MSN output.

PV-INS are identified electrophysiologically by (a) low input resistance (50-150 MΩ), (b) narrow, high frequency action potentials with a maximal sustained firing rate at 200 Hz, (c) large-amplitude spike afterhyperpolarization potentials without adaptation, and (d) a linear current-voltage (I-V) relationship. The voltage-gated K⁺ channel, Kv3.1, colocalizes with PV in fast-spiking INs in the cerebellum, hippocampus, globus pallidus and dStr of the basal ganglia. Kv3.1 differs from related delayed-rectifying Kv channels in that it activates rapidly and deactivates slowly at high-threshold potentials (8-10x faster), indicating that Kv3.1 may contribute, in part, to the kinetics of high-frequency action potentials in PV-INS. A subpopulation of PV-INS in the dStr has also been shown to exhibit subthreshold oscillatory changes in membrane potential that interrupt periods of “chattering” spike activity, a pattern eliminated by the voltage-gated Na⁺ channel blocker, tetrodotoxin. The mechanism by which these properties ultimately regulate circuit function in the NAc, however, remains to be determined.

Inhibitory postsynaptic currents (IPSCs) generated by PV-INS in D1R and D2R MSNs are abolished in the presence of picrotoxin, a non-selective GABA₂ receptor antagonist, indicating that PV-INS-to-MSN GABAergic transmission occurs predominately via GABA₂R. GABA₂R is a heteropentameric receptor most commonly comprised of two α, two β, and one γ or δ subunit, the stoichiometry of which dictates receptor function, location, and pharmacosensitivity. The exact pentameric subunit composition of GABA₂Rs in the NAc is unclear, although α₁- and α₂/α₄-containing GABA₂Rs have been shown to be expressed preferentially on INs and MSNs, respectively. Unitary recordings indicate that PV-INS-to-MSN signaling occurs at perisomatic and proximal dendritic sites, exerting robust, high-amplitude inhibitory control over MSN spike activity. In contrast, triple-positive IN- and MSN-to-MSN synapses occur at distal dendritic domains, producing smaller IPSCs that may sparsely neutralize glutamatergic input in the same region.

Perhaps the most significant function of PV-INS in striatal circuit function is their involvement in feedforward inhibition, where afferent excitatory input onto MSNs from diverse limbic-related (BLA, vHipp, mPFC) cortical structures collateralizes onto adjacent PV-INS. The temporal delay between afferent excitatory signaling and GABAergic input from PV-INS creates a dynamic window regulating MSN output. GABAergic signaling at PV-INS-to-MSN synapses has been shown to exert stronger and more extensive inhibitory control over MSN output than lateral inhibition from recurrent MSN collaterals, indicating that feedforward inhibition from PV-INS may serve as the primary mechanism regulating NAc output. Furthermore, computational models in the dStr suggest that PV-INS more strongly influence D2R MSN activity than D1R MSN. While the same has not been demonstrated in the NAc, biased GABAergic signaling at D2R MSNs...
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in the NAc has profound implications for drug-induced synaptic rearrangements that enhance excitatory drive onto D1R MSNs.

**CB1R-dependent eCB signaling mechanisms in the NAc and their role in synaptic plasticity**

Endocannabinoid signaling has been shown to modulate GABAergic signaling in various brain regions, including the NAc, hippocampus, dStr, and cerebellum. The two primary eCBs, anandamide (N-arachidonoyl-ethanolamine, AEA) and 2-arachidonoylglycerol (2-AG), are lipophilic retrograde messengers synthesized and released postsynaptically under depolarizing conditions. 2-AG is synthesized from diacylglycerol (DAG) by DAG lipase-α, whereas NAPE-specific phospholipase D converts N-acylphosphatidylethanolamine (NAPE) to AEA. eCBs mobilized from postsynaptic neurons travel retrogradely across the synapse to bind to CB1R expressed on presynaptic terminals. At inhibitory terminals, CB1R activation decreases presynaptic neurotransmitter release probability at short and long timescales, referred as depolarization-induced suppression of inhibition (DSI) and eCB-dependent inhibitory long-term depression (eCB-iLTD), respectively. eCB-iLTD differs from DSI further in that its effects persist in the absence of CB1R-ligand interaction and engages different intracellular targets.

CB1R is a Gαi/o-coupled G-protein-coupled receptor (GPCR) encoded by the cannabinoid receptor 1 (CNR1) gene that signals through Gαi and Gβγ subunits. While CB1R is expressed richly on interneurons and MSNs in the dStr, its expression in the NAc is restricted to PV-INS and glutamatergic afferents, explaining its relatively poor immunoreactivity in the NAc. Short-term (sec-min) reduction of presynaptic neurotransmitter release has been shown to occur via voltage-gated Ca2+ channels and inward-rectifying potassium channels (Kir) at presynaptic active zones, preventing action potential-dependent neurotransmitter release. In contrast, Gβγ-mediated inhibition of adenyl cyclase activity purportedly mediates long-term (hours) plasticity by reducing cAMP and PKA levels. Despite considerable evidence that eCBs signal predominately through CB1R in the CNS, recent reports indicate that AEA triggers a postsynaptic, AMPAR-dependent form of LTD via transient vanilloid (TRPV1) receptors, indicating that eCBs may have a more diverse loci of action than previously thought.

DSI has been illustrated at PV-IN-to-MSN synapses in the NAc but not PV-IN-to-PV-IN or MSN-to-MSN synapses, indicating that CB1R activity on PV-INS putatively regulates GABAergic input onto MSNs. Hippocampal and dStr DSI is absent in global DAG lipase-α knockout mice and unaltered in acute brain slices treated with an inhibitor of AEA hydrolysis, indicating that 2-AG is the predominant eCB mediating DSI in these regions. In the hippocampus, transient CB1R activation disrupts γ-frequency (30-100 Hz) oscillations that are important for memory-related processes, a finding pertinent to the NAc given that PV-IN spike activity is phase-locked with γ-frequency oscillations. DSI of GABAergic transmission at PV-IN-to-MSN synapses in the NAc may transiently disinhibit MSN output by increasing heterosynaptic responsiveness to excitatory input, a form of metaplasticity well-characterized in the VTA, hippocampus and amygdala.

The induction and maintenance of eCB-iLTD is more complex, with cell-type and state-dependent induction mechanisms that differ across brain regions. 2-AG mediates electrically evoked (1 Hz, 80 sec) eCB-iLTD at D1R MSNs in the dStr when held in their upstate, whereas AEA mediates eCB-iLTD at D2R MSNs in their downstate. In contrast, eCB-LTD of glutamatergic synapses (10 Hz) in the NAc appears to be restricted to D2R MSNs and is metabotropic glutamate receptor (mGluR2)-dependent. The complexity of eCB signaling is illustrated in studies showing that GABAergic terminals in the dStr are more sensitive than glutamatergic terminals to CB1R-dependent plasticity mechanisms. Low-frequency induction, for example,
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generates robust eCB-ilTDr of GABAergic inputs that is insufficient to enact similar changes in glutamatergic inputs. Furthermore, studies in the dStr mirror those in the hippocampus and BLA showing that eCB-ilTDr necessitates heterosynaptic group I mGluR activation. Together, DSI and iLTD in the NAc likely recruit disparate molecular mechanisms that may underlie drug-related behavioral states.

Putting it all together: What role does eCB signaling and inhibitory neurotransmission have on drug-related behavior?

The eCB system has been extensively implicated in drug-induced synaptic and behavioral adaptations. CB,RR activation on GABAergic INs in the VTA has been shown to augment mesoaccumbens DA output. Δ⁹-tetrahydrocannabinol (Δ⁹-THC), the primary psychoactive agent in marijuana, is a potent CB,R agonist that purportedly increases phasic DA release by inducing eCB-ilTDr at INs in this location. Furthermore, acute Δ⁹-THC administration increases the AMPAR/NMDAR ratio at glutamatergic afferents in the VTA, indicating that CB,R activity on GABAergic INs “primes” NMDAR-dependent long-term potentiation (LTP) to take place at excitatory synapses. Enhancement of eCB-ilTDr and LTP at excitatory synapses onto DA-expressing projection neurons in the VTA has also been shown in cocaine, morphine, and nicotine-treated mice.

In the NAc, acute Δ⁹-THC exposure abolishes eCB-LTD at D1R and D2R MSNs by desensitizing CB,R activity at presynaptic terminals. This effect emerges following a single injection and persists during a 7-day regimen. Similarly, in vivo cocaine exposure abolishes eCB-LTD at D2R MSNs in a mGluR-dependent manner. mGluR₅ is a G₁₅₁-coupled GPCR expressed at perisynaptic sites that mobilizes eCB release. Cocaine has been shown to sequester mGluR₅ by disrupting its interaction with Homer, a scaffolding protein that links membrane-bound mGluRs to intracellular proteins. This finding also supports the glutamatergic hypothesis of relapse, which states that cued reinstatement of drug seeking is intensified following cocaine-induced remodeling of excitatory synapses in the NAc. Abstinence from cocaine self-administration results in a time-dependent increase in excitatory synaptic strength at vSub and mPFC-MSN synapses. Insertion of high-conductance, GluA2-lacking Ca²⁺-permeable (CP)-AMPARs has been shown to mediate this effect. Therefore, impaired eCB-LTD at glutamatergic synapses may enhance excitatory drive onto MSNs during withdrawal.

The importance of NAc-specific GABAergic neurotransmission in drug-induced synaptic adaptations has only recently garnered interest. Five-days of intraperitoneal (IP) cocaine exposure has been shown to increase PV-IN membrane excitability to intracellular current injection, indicating that inhibitory signaling at PV-IN-to-MSN synapses may be increased following acute psychostimulant exposure. This is supported by data showing that cocaine re-exposure following abstinence increases miniature IPSC frequency at MSNs in the NAc. Miniature IPSC frequency and amplitude measurements reflect pre- and postsynaptic changes, respectively. Furthermore, early abstinence from limited-access cocaine exposure increases surface expression of α₆-containing GABA₆,R on MSNs. Intra-NAc stimulation of this GABA₆,R isoform blocks cocaine-induced conditioned place preference (CPP). While PV-INs exhibit enhanced excitability following cocaine exposure, electrically-evoked excitatory-inhibitory (E/I) balance of synaptic inputs onto CB,R-expressing PV-INs in the NAc remains unchanged, suggesting that changes in GABAergic signaling in the NAc likely arise from intrinsic differences in interneuron membrane properties and postsynaptic responsiveness to GABA.

Despite a paucity of data looking at cocaine-induced changes in GABAergic input onto D1R and D2R MSNs, these findings coincide with cocaine-generated synaptic rearrangements in the NAc. For example, acute contingent cocaine exposure attenuates MSN output by (a) increasing GluN2B-containing NMDAR-only
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silent synapses, (b) decreasing extrasynaptic glutamate by impairing system-x, cystine-glutamate exchange, and (c) decreasing intrinsic MSN excitability. Increased PV-IN-to-MSN signaling following cocaine exposure may reduce MSN spike threshold further by hyperpolarizing the cell via GABA<sub>A</sub>R-mediated Cl<sup>-</sup> influx. PV-INs are probable candidates morphologically in that their axon collaterals breach patch (striosomal)-matrix boundaries, synchronizing MSN responsiveness throughout the NAc. The relationship between MSN output and GABA is highlighted further by data showing that mIPSC amplitude decreases during abstinence from cocaine, a time-point at which CP-AMPAR-mediated EPSCs is greatest.

While the mechanism by which Δ<sup>2</sup>-THC and other cannabinoids alter synaptic physiology in the NAc is incompletely understood, the role of CB<sub>1</sub>R in addiction behavior has been intensely studied. Polymorphisms in the human CB<sub>1</sub>R-encoding CNR1 gene, for example, have been linked to polysubstance abuse disorders. Several studies have shown that IP Δ<sup>2</sup>-THC analogs have no effect on self-administration of cocaine, an operant paradigm in which animals perform a task (lever press) to receive a reward (cocaine). CB<sub>1</sub>R blockade with AM-251 or rimonabant, CB<sub>1</sub>R inverse agonists, and CNR1 knockout mice also fail to prevent behavioral sensitization to cocaine, indicating that eCB signaling is likely uninvolved in the rewarding and behavioral effects of contingent cocaine exposure. Interestingly, rimonabant impairs morphine-induced CPP, a measure of drug-induced reward learning. Global CNR1<sup>-/-</sup> mice exhibit blunted morphine self-administration, indicating that CB<sub>1</sub>R is differentially involved in the rewarding properties of cocaine and morphine. WIN 55-212, a synthetic CB<sub>1</sub>R agonist, reinstates cocaine-, morphine-, and notice-seeking behavior in mice, an effect that is abolished in AM-251-treated cohorts. These data indicate that CB<sub>1</sub>R activity in the mesolimbic system underlies drug-specific behavioral adaptations.

Conclusion

The NAc mediates complex motivational behavior by integrating diverse synaptic inputs from limbic-related brain structures. It is thought that drugs of abuse attain their addictive potential by modifying synaptic events at these inputs. While dopaminergic and glutamatergic signaling in the NAc has been extensively characterized, little is known regarding GABAergic signaling at PV-IN-to-MSN synapses and how eCB regulation of these synapses shapes NAc-dependent motivational behavior. Despite making up only 0.7-1% of neurons in the NAc, a single, electrically-coupled PV-IN can exert robust inhibitory control over up to 100 MSNs, indicating that PV-INs may have a more important role in NAc circuit function than previously thought. PV-IN-to-MSN microcircuits are also the only known inhibitory synapses in the NAc to undergo CB<sub>1</sub>R-dependent plasticity. Provided the importance of eCBs in mesolimbic circuit function, further studies are needed to determine the relationship between eCBs and PV-INs and their role in addiction-related pathology.

References

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Physiological Stress Systems and the Influence of Physiology on Behavior and Affect in Autism Spectrum Disorder (ASD)

Rachael A. Muscatello

Abstract

Stress can be defined as disruption of homeostasis or threat to well-being, and sources of stress can be real or perceived, physical or psychological. The hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous system (ANS) both serve to regulate physiological arousal during times of stress. These collective systems function independently, yet they share significant overlapping circuitry in neural regions involved in the perception of and response to environmental changes and stressors. These circuits are also involved in social and emotion regulation, and dysfunction of the stress response may contribute to social deficits and psychopathology. Individuals with autism spectrum disorder (ASD) exhibit core impairment in social communication as well as prevalent co-occurring internalizing symptoms such as anxiety and depression. Many individuals with ASD experience an atypical stress response to environmental changes, including novel social interactions; therefore, ASD may serve as a useful model for understanding the role of stress neurocircuitry in social behavior and affect. This furthered understanding may provide insight into how patterns of physiological arousal can contribute to notable differences in behavior.

Keywords: hypothalamic-pituitary-adrenal axis, autonomic nervous system, autism spectrum disorder, stress, social engagement, internalizing disorders

Introduction

The experience of stress and arousal is a common, everyday occurrence for all humans, and appropriate physiological and psychological responses are necessary for adaptive interactions with the environment. Stress can be difficult to objectively define, but for the context of this review, the term 'stress' will refer to the body’s physiological response to threat. This physiological response is efficient, highly conserved and serves to minimize metabolic cost. Two important systems for managing and responding to stress and arousal include the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS). The HPA axis is primarily involved in physiological reactivity in response to stress, while the ANS acts more as a physiological regulator. While both systems function independently from one another, there is also considerable overlap and interconnection between these systems, which serves to maintain appropriate response to a variety of environmental demands. Due to the substantial influence of these systems on both physiological and psychological functioning, dysfunction of the HPA axis and/or ANS may have significant negative consequences for a wide spectrum of behaviors.

The Hypothalamic-Pituitary-Adrenal (HPA) axis

The HPA axis is the primary neuroendocrine stress system, secreting glucocorticoids, such as cortisol, from the adrenal glands. During activation, the paraventricular nucleus of the hypothalamus (PVN) will release corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), which stimulate release of adrenocorticotropic hormone (ACTH) by binding to receptors on corticotropic cells of the anterior pituitary.
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ACTH then binds to receptors at the adrenal cortex, leading to release of glucocorticoids, including cortisol. While cortisol will be released in response to stress, HPA regulation also includes maintaining homeostasis and restoring basal cortisol levels through negative feedback mechanisms by which cortisol itself will inhibit further release of CRH by the PVN. Cortisol is the primary human glucocorticoid. Previous research has shown that concentrations can be reliably measured through saliva, and salivary cortisol concentrations are highly correlated with plasma cortisol. Therefore, salivary cortisol is considered a useful noninvasive biological marker of stress in human behavioral studies.

Neuroendocrine response to stress arises from two separate circuits, based upon the type of stressor. The first involves response to immediate physiological threat (‘systemic’ stressors), and the response is quickly activated by stimulation of the PVN. A second type of stressor, however, relies greatly on perceived threat within the environment and processing of contextual, emotional, and memory-based cues. This processing of perceived, psychological stress (‘processive’ stressors) is heavily mediated by a variety of limbic structures crucial in regulating the stress response. Connections with many higher-order processing structures, such as the prefrontal cortex, the amygdala, and the hippocampus, serve an important role in activating or inhibiting HPA response to these psychological stressors.

There are four features of psychological stressors that are known to activate the HPA axis: novelty, unpredictability, social evaluation, and a sense of low control. Early studies of rodents and non-human primates revealed a strong effect of novelty, with even minimal exposure to new environments leading to a measurable increase in plasma cortisol secretion. Many studies in humans have shown threats to self-preservation or to the social self, such as in a laboratory-based social evaluative threat paradigm, will lead to a significant increase in salivary cortisol secretion, especially when the situation is perceived as uncontrollable. More benign social situations that do not threaten the social self are not expected to lead to increases in cortisol response. If cortisol reactivity is high in contexts which are not typically perceived as stressful or threat-inducing, this may suggest dysfunction of the HPA axis.

Altered HPA functioning, including both changes in the diurnal rhythm or in response to stressors, has been implicated in psychopathology, including increased incidence of internalizing symptoms. While early rodent and non-human primate studies have shown that acute stress exposure early in life does not necessarily lead to dysfunction of the HPA axis in adulthood (see for review), chronic exposure to multiple stressors and an imbalanced physiological response can lead to increased allostatic load. Allostatic load, or overload, can be defined as “the cumulative pathophysiology that can result from this [HPA axis] dysregulation and excess stress” (p. 1353). Acute and chronic stress exposure may lie on an inverted-U shaped dose/response curve, where some moderate acute and chronic stress may be adaptive, which is exemplified in an early study showing rat pups that were occasionally separated from the mother were more resistant to stress and HPA reactivity later in development. Severe acute or chronic stress, however, can lead to excitotoxicity, mediated by glutamate and glucocorticoids, contributing to impaired synaptic remodeling, loss of resilience or plasticity, and increased vulnerability to further stress exposure. According to the neurotoxicity hypothesis, this chronic stress exposure may lead to significant neuronal damage to regions implicated in cognitive and emotional regulation, such as the hippocampus, prefrontal cortex, and amygdala.

Accumulation of stress throughout the lifespan and allostatic overload can have significant implications for mental health, including early risk of depression and anxiety in children and adolescents. Empirical evidence from human behavioral studies has shown increased cortisol reactivity is associated with more anxiety and depressive symptoms in older female children, as well as altered stress recovery following social evaluation in adults with major depressive disorder. Moreover, there is significant evidence to suggest that altered diurnal patterns of the HPA axis may predict internalizing symptoms in children and adolescents.
The Autonomic Nervous System (ANS)

The ANS regulates visceral organs, including the heart. Projections from the ANS are separated into two branches, the parasympathetic (PNS) and sympathetic (SNS) nervous systems, which have distinct functions within the body. In general, the PNS mediates a ‘rest and relax’ response, while the SNS is described as the ‘fight or flight’ system. Efferent projections from both the PNS and the SNS can have immediate action on the heart via innervation of the sinoatrial (SA) node. The PNS slows heart rate via cholinergic output of the vagal nerve (cranial nerve X) binding to muscarinic receptors of the SA node. The SNS, in contrast, releases norepinephrine from postganglionic nuclei, speeding heart rate via a beta-adrenergic receptor-mediated second messenger cascade.

Central autonomic control originates from many regions, including those in the brainstem, hypothalamus, and limbic forebrain. Brainstem regions, especially within the medulla, control vagal tone and respiration and include the nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus (DMX) and nucleus ambiguus (NA). The NTS serves as a relay station for sensory information carried by the vagus and other regions, such as the insular cortex. Sensory information is also sent to other central regions, such as the amygdala, prefrontal cortex, and hypothalamus. These forebrain regions send downstream signals to the brainstem, leading to ANS output and stimulation of the appropriate effector organs (see Figure 1). Certain regions within the central autonomic network are specifically related to parasympathetic stress response, including primary output from the DMX and NA, which are generally influenced by the NTS and paraventricular nucleus of the hypothalamus (PVN). The SNS stress response, however, is activated by limbic and hypothalamic regions such as the amygdala, which send signals to the ventrolateral medulla (VLM), thereby leading to catecholaminergic output by the postganglionic SNS.

Heart rate variability (HRV) can serve as a useful marker of changing parasympathetic and sympathetic activity. Two commonly utilized non-invasive measures of cardiac ANS response include respiratory sinus arrhythmia (RSA) and pre-ejection period (PEP). RSA is an indicator of the activity of the parasympathetically-driven vagus nerve at the SA node, and thus serves as a measure of PNS regulation. RSA is further defined as a measure of the high-frequency component of HRV in conjunction with respiratory patterns. The most valid measure of sympathetic cardiac control is PEP, the length of time from stimulation of the left ventricle to opening of the aortic valve. Therefore, a decrease in time to contraction is indexed by a lower PEP, which indicates greater SNS activation.

The ANS has been proposed as a primary behavioral regulator, and the balance between the PNS and the SNS may be crucial in regulating response to stress. According to the Polyvagal Theory on autonomic arousal, the myelinated vagus evolved as the primary stress response system in mammals. This perspective argues the human stress response is driven by the vagal system, and during times of restful conditions, the PNS stays active and maintains a ‘vagal brake’ on the heart. During stress, however, vagal tone decreases (vagal withdrawal), which allows for rapid increases in cardiac output without engagement of the SNS. According to Porges, this vagal flexibility is crucial for fast response to environmental stressors while preventing excessive SNS reactivity. In times of increasing threat, however, the SNS will become active, initiating the fight or flight response. This activation requires significant metabolic resources, and it is now clear from extensive animal and human studies that chronic heightened reactivity can have significant negative impacts on physical and mental health. Therefore, the balance of the ANS is crucial not only for appropriate physiological response to environmental demands, but also for preventing potential detrimental outcomes resulting from an unbalanced stress response.
According to the Polyvagal Theory\textsuperscript{3}, appropriate ANS balance and vagal flexibility is also necessary in determining social behavior. The parasympathetically-mediated social engagement system primes the system for interaction with the environment through shared connections between the vagus and cranial nerves involved in regulation of muscles of the face and head\textsuperscript{44-46}. Any activation of the SNS will promote mobilization behaviors, overriding the vagal brake and making the components of the social engagement system relatively inaccessible\textsuperscript{44}. Therefore, according to this theory, proper functioning of the PNS and the vagal brake is required for appropriate social functioning, and any dysfunction of this system would compromise social behavior\textsuperscript{46}.

An imbalanced ANS may contribute to negative mental health outcomes. For example, consistently heightened SNS reactivity is associated with both internalizing and externalizing symptoms\textsuperscript{51}. Similarly, low HRV, which is indicative of low PNS influence and more SNS reactivity, has been found in depression\textsuperscript{52}, generalized anxiety disorder\textsuperscript{53}, and poor emotion regulation\textsuperscript{54}. One explanation for this relationship between HRV and emotion regulation arises from the view that affect reflects one's ability to adjust to constantly changing environmental demands\textsuperscript{55}. A fast-acting, flexible system is required in order to match affective response with the situation. Moreover, central control of the ANS can play a significant role in determining behavioral response. For example, the prefrontal cortex inhibits structures associated with defensive behaviors, such as the amygdala, as well as inhibiting sympathoexcitatory circuits. If any part of this pathway is disrupted, thus removing tonic inhibition, a rigid pattern emerges which promotes sustained activation of mobilization responses\textsuperscript{55}. Rigidity of this defensive system may in turn promote hypervigilance or perseveration of cognition and negative affect, thereby promoting dispositions associated with anxiety and depression\textsuperscript{55,56}.

**Integrated response of the HPA axis and ANS: A Dual System Approach**

Understanding how the HPA axis and ANS interact and work together during stress and recovery may be key to elucidating the impact of dysfunction of one or both systems on cognition and behavior, especially in regards to social behavior and emotion regulation. According to the model of Neurovisceral Integration (NVI)\textsuperscript{37-59}, a network of brain regions, many of which are associated with the central autonomic network (CAN)\textsuperscript{39}, interact to have downstream regulatory effects on autonomic and cognitive processes. Specifically, shared connections (as shown in Figure 1) with regions such as the prefrontal cortex, amygdala, and hypothalamus may act as an internal regulation system to link the HPA axis and ANS\textsuperscript{55}.

During stress, the HPA axis and the ANS have complementary and overlapping responses. While the signaling cascade of the HPA axis originating from the PVN ultimately stimulates cortisol release from the adrenal cortex, the sympathetic system also directly innervates the adrenal cortex and can stimulate cortisol release, thereby providing further evidence of complementary HPA and SNS actions to mobilize energy resources in response to stress\textsuperscript{8}. The PNS, however, tends to have opposing functions within the body, as shared regions which stimulate the SNS and HPA axis will inhibit downstream circuitry for the PNS during a stress response\textsuperscript{47,55}. For example, when the PVN of the hypothalamus is activated in response to a stressor, it will send excitatory signals to the SNS as well as activating the cascade of the HPA axis to release cortisol. Activation of the PVN will similarly lead to inhibitory outputs on the dorsal motor nucleus of the vagus and the nucleus ambiguus, thus resulting in decreased vagal tone and reduced PNS inhibition on the cardiac system\textsuperscript{2,58}. The combined activation of the SNS and HPA axis, in concert with inhibition of the PNS, will result in a stress response system which is primed to promote mobilization for fight or flight behaviors\textsuperscript{3,57}. 
Figure 1. Representation of overlapping neurocircuitry of physiological stress systems.

Regions involved in regulating both the HPA axis (green arrows) and the ANS (blue arrows). The simplified representation illustrates stress circuitry, including shared central regulatory regions (purple arrows), beginning with top-down modulation from the prefrontal cortex, as well as regulatory inputs from limbic regions such as the hippocampus and amygdala, and ending with output at the various regions downstream.

ACTH- adrenocorticotropic hormone; AVP- arginine vasopressin; CRH- corticotropin-releasing hormone; DMX- dorsal motor nucleus of the vagus; LC- locus coeruleus; NA- nucleus ambiguus; NTS- nucleus tractus solitarius; PNS- parasympathetic nervous system; PVN- paraventricular nucleus of the hypothalamus; SNS- sympathetic nervous system; VLM- ventrolateral medulla.

Shared regulatory brain regions of the HPA axis and the ANS also serve to indirectly connect the two systems, as few direct connections between the two stress systems exist. These regions provide top-down modulation of the stress response, especially in regards to response to psychological stressors. This corticocortical circuit consists of overlapping connections with many regions implicated in social, emotional, and cognitive responses including the amygdala, hippocampus, and medial prefrontal cortex. For example, the amygdala is often described as the ‘threat-detector’ and activates during a fear response. Because the amygdala sends direct and indirect projections to the HPA axis, the SNS, and the PNS (Figure 1), activation of the region will have downstream effects on all stress systems. The amygdala is also reciprocally connected with the hippocampus, thus providing a system by which previous memories of threat or safety may influence perception of environments as safe or novel. In situations of threat and/or novelty, the amygdala can send excitatory output to the HPA axis and the SNS via connections with the NTS and PVN. Additionally, GABAergic signaling from the amygdala will inhibit NTS signaling to the vagus (via the nucleus ambiguus), thus leading to downregulation of the PNS.
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The prefrontal cortex plays a major role in top-down modulation of the stress response. The prefrontal cortex maintains inhibitory control on the excitatory stress circuitry, such as through inhibition of the amygdala. This tonic inhibition generally prevents the stress response; however, during times of uncertainty or perceived threat, the prefrontal cortex will be hypoactive, leading to disinhibition of the sympathetic and endocrine stress circuits. Additionally, the prefrontal cortex receives inputs from other cortical and subcortical structures, such as the hippocampus and amygdala, which are involved in memory and context-dependent conditional behavioral responses to emotional stimuli. These extensive interconnections between limbic forebrain regions, with the prefrontal cortex at the top of the response hierarchy, serve to maintain top-down regulation on stress circuitry, contributing to a context-specific response based upon cognitive appraisal of the current environment.

Given the top-down modulation involved in the neurocircuitry of stress, disruption of this circuitry is clearly implicated in psychopathological conditions, including affective disorders, such as anxiety and depression. For example, if the prefrontal cortex becomes hypoactive, the sympathoexcitatory circuits are inhibited, leading to prolonged energy mobilization states and biased attention for threat information. Moreover, according to the Polyvagal theory, the vagally-mediated parasympathetic nervous system has evolved to become the first and primary system to respond to differences in the environment, and during times of little to no stress, the PNS will stay active and inhibit cardiac output. Appropriate response to a variety of situations relies upon flexibility of the vagus and the PNS, and if this system becomes hypoactive or dysfunctional, inhibition of the more energy-demanding sympathetic and HPA stress systems will be reduced, leading to energy mobilization and increased allostatic load. As these chronic, hyperactive stress responses have been implicated in internalizing disorders, it becomes evident that proper regulation and interactions between these systems is likely essential for appropriate behavior.

Physiological Arousal Mediates Behavior: Autism Spectrum Disorder (ASD) as a Model

Autism spectrum disorder is a neurodevelopmental disorder characterized by impairments in two core diagnostic domains, social communication and restricted, repetitive behaviors and interests. Children and adolescents with ASD often experience increased psychological stress, especially resulting from difficulty tolerating change and novelty. Difficulties in reciprocal social interaction and increased stress are often related in ASD, as children with ASD who do engage in social interactions frequently find these events to be stressfull and anxiety-inducing. Comorbid internalizing disorders in youth with ASD are especially prevalent. One challenge in ASD is that assessment of depression or anxiety symptoms typically relies upon self- or parent-report, which can be challenging in a population that may have difficulty verbalizing emotions or having insight into their internal states (see for review). The same may be said for symptoms of stress, which argues for the need for more objective measures of stress symptoms in ASD.

An avenue of increased interest lies in the apparent connections between social functioning, stress, internalizing symptoms, and physiological arousal. As reviewed above, both the HPA axis and the ANS can influence social functioning and the stress response. Moreover, recent interest has focused on using physiological markers as not only a measure of stress, but also as a means to potentially predict internalizing symptoms, which are frequently accompanied by somatic manifestations such as increased heart rate. By recognizing the apparent role of physiological stress systems in mediating social behavior and emotion regulation, as well as noting the many impairments in these systems within ASD (see for review), ASD serves as an ideal pathological model to implicate physiological measures as a biological marker of stress, social functioning impairment, and internalizing symptoms in humans.
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There is significant evidence for HPA axis dysfunction in children with ASD, including both altered diurnal rhythm and hyper- or hypo-responsivity to various psychological stressors (see 79 for review). Previous studies have shown lower morning cortisol\(^{80,81}\) and elevated evening cortisol values\(^{81-84}\), thereby leading to an overall blunted diurnal rhythm in at least a subset of children with ASD\(^{82,83}\). Moreover, these studies have found significant relationships between dysfunction of the diurnal rhythm and other symptoms including stress\(^{81}\), sensory sensitivity\(^{81}\), anxiety\(^{85}\), and depression\(^{86}\).

Individuals with ASD also demonstrate atypical HPA axis reactivity to stress. During a naturalistic playground interaction\(^{68}\), many children with ASD have an elevated cortisol response relative to typically developing (TD) peers, suggesting they perceive these interactions as especially stressful\(^{86,70,87}\). Moreover, heightened cortisol is associated with less cooperative play with peers in some children\(^{70}\). Interestingly, while children with ASD show this increased cortisol reactivity to benign social interactions, the opposite holds true for prototypical stressors, such as in cases of social judgement. One widely used social evaluative stressor is the Trier Social Stress Test (TSST)\(^{17}\), which is known to lead to increased cortisol responsivity in TD children\(^{18,19}\); however, the task frequently fails to elicit a stress response in youth with ASD\(^{88-91}\). These findings reflect an apparent failure of the HPA axis in children with ASD to activate in response to typically stressful events, such as social evaluation, yet a significant hyperarousal during relatively benign, non-threatening social interactions.

In addition to dysfunction of the HPA axis, individuals with ASD may experience autonomic dysfunction, although the nature of these impairments remains unclear as previous research has produced conflicting results. While many studies have found individuals with ASD have reduced parasympathetic regulation at baseline relative to TD individuals\(^{92-95}\), other studies have found no baseline difference\(^{90,96,97}\). The few studies to date that have measured SNS activity using PEP have found no differences during a baseline rest period\(^{98,99}\). In regards to reactivity of the ANS, children with ASD, compared to TD peers, have shown lower RSA and greater decrease in RSA (decreased parasympathetic regulation) when interacting with unfamiliar persons. In contrast, greater PNS regulation was associated with better social skills and fewer problem behaviors\(^{93}\). During social evaluative threat, adolescents with ASD had greater RSA withdrawal (decrease in parasympathetic activity) compared to TD adolescents\(^{93}\). Moreover, greater RSA withdrawal was associated with more social problems. These findings provide some support for the Polyvagal Theory of a social engagement system\(^{16}\), such that altered vagal functioning, including improper withdrawal of inhibition or inflexibility during stress, may directly impact social behaviors in ASD\(^{98,100}\).

While current findings are limited, there is evidence to suggest that children with co-occurring ASD and anxiety have a blunted cortisol response to cognitive and psychosocial stressors\(^{101-103}\). Similar findings have been seen in studies of the ANS response to stress, which have shown that blunted heart rate and electrodermal response (sympathetic activity) are associated with greater anxiety in children with ASD\(^{101,102,104-106}\). Others, however, have not found associations between cortisol and anxiety in ASD\(^{69,89,107}\), or between ANS response and anxiety\(^{96,108}\). It is important to also consider the potential effects of interactions between the HPA axis and the ANS, as a few studies have investigated how these interactions may relate with internalizing symptoms in neurotypical children\(^{51,109,110}\). These studies show that greater PNS activity during a resting baseline state may be protective in terms of lowered risk for internalizing symptoms\(^{109}\), while higher basal cortisol and high SNS activity are associated with significantly increased internalizing symptoms\(^{51}\). Hyperarousal across multiple systems in conjunction with a reduced ‘vagal brake’ may be associated with increased risk for anxiety and/or depression. Given the prevalence of internalizing symptoms in ASD, as well as the complexity of relationships between the HPA axis, ANS, and affect, a more thorough approach that considers multiple physiological stress systems and their patterns of interaction is necessary.

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Conclusions

The HPA axis and ANS stress responses are heavily implicated in social and emotional functioning. While they are independent systems, the HPA axis and ANS circuitry share a significant amount of overlap, and it is these shared connections that may have implications for increased risk of behavioral and affective dysfunction. As previous findings have shown, children with ASD frequently display dysfunction of the HPA axis and ANS responses to stress, as well as increased prevalence of anxiety and depression. Given the noted connections between the stress systems, as well as their roles in mediating social and emotional behavior, it stands to reason that alterations in the neurocircuitry of stress in ASD may underlie or contribute to many of the noted symptoms related to the ASD diagnosis.

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The role of NKCC1 in regulating Cl⁻ levels and cell volume homeostasis in neurons and smooth muscle cells

Salma Omer

Abstract

The health of most cells in our body depends on the proper functioning of homeostatic mechanisms that regulate ions, including Cl⁻, and water. Regulation of intracellular Cl⁻ in the nervous system and vasculature is largely mediated by the family of cation-coupled chloride transporters (CCCs). Sensory afferent neurons benefit from a large outward Cl⁻ gradient affecting pain/sensorimotor function, where Cl⁻ efflux leads to primary afferent depolarization (PAD) mediated by GABA. Likewise, in smooth muscle cells, an outward electrochemical Cl⁻ gradient leads to membrane depolarization, activation of voltage-sensitive Ca²⁺ channels, cell contraction and increased vascular tone. A common denominator in the regulation of intracellular Cl⁻ in these cell types is the Na⁺-K⁺-2Cl⁻ cotransporter-1 (NKCC1). The high expression of NKCC1 in cells increases the intracellular Cl⁻ concentration above the electrochemical potential equilibrium thereby creating an outward gradient that cells use to their advantage. Although NKCC1 is ubiquitously expressed and NKCC1 knock-out mouse models display disease phenotypes, there was until recently no documented human disease associated with NKCC1 dysfunction other than temporary deafness associated with high doses of loop diuretics. This indicates either that NKCC1 function has built-in redundancy, or that loss of function mutations are not well tolerated in the human population. Because disruption in Cl⁻ homeostasis has been implicated in several human diseases, further characterization (or understanding) of these channels is critical.

Keywords: NKCC1, Cell volume regulation, choroid plexus, inner ear, dorsal root ganglia, pain pathway, smooth muscle cells

Introduction: cation-coupled chloride transporters

The tight regulation of intracellular Cl⁻ concentrations ([Cl⁻]) is critical for the function of many physiological processes including cell volume regulation, modulation of intra-neuronal Cl⁻ concentration, ion movement across epithelial cells, and blood pressure regulation¹. Mutations in genes involving the transport of Cl⁻ have been linked to inherited as well as non-inherited forms of human disease. The SLC12 gene family, which encodes electroneutral cation-coupled chloride transporters (CCCs), is involved in the regulation of Cl⁻, as well as Na⁺ and K⁺. The SLC12 gene family is divided into two main branches based on their sensitivity to pharmacological inhibitors, their stoichiometry of transported ions, and their phylogeny²: the 1) Na⁺ independent and K⁺ coupled Cl⁻ cotransporters (KCCs) and the 2) Na⁺ dependent Cl⁻ cotransporters (NCC and NKCCs).

The CCCs are membrane proteins that primarily function to transport Cl⁻ ions along with Na⁺ and/or K⁺ ions across the plasma membrane of cells. The large transmembrane Na⁺ and K⁺ gradients established by the Na⁺-K⁺-ATPase energetically drive these transporters. The CCCs transport an equivalent number of positive and negative ions across the membrane, and so transport is electroneutral. The large electrochemically-favorable inward gradient for Na⁺ works in the favor of Na⁺ coupled chloride cotransporters and allows them to transport Cl⁻ into the cell, raising [Cl⁻] above the Cl⁻ electrochemical equilibrium or reversal potential (ECl⁻).
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Similarly, the K⁺ coupled chloride cotransporters take advantage of the outward gradient for K⁺ by transporting Cl⁻ out of the cell, reducing [Cl⁻]ᵢ below E₃Cl⁻. The degree to which each transporter is active in mediating Cl⁻ efflux or influx determines the [Cl⁻]ᵢ in various cell types, resulting in the regulation of neuronal excitability, cell volume, and transport of sodium, potassium, and water across epithelia.

The CCC family of transporters are subject to regulation by extracellular conditions and by phosphorylation. Specifically, extracellular hypotonicity triggering cell swelling, high [Cl⁻]ᵢ, and protein phosphatases (PPs) involved in cotransporter dephosphorylation activate the KCCs and inhibit the NKCCs, thereby decreasing [Cl⁻]ᵢ and resulting in regulatory volume decrease (RVD) of the cell. Conversely, extracellular hypertonicity triggering cell shrinkage, low [Cl⁻]ᵢ, and kinases involved in cotransporter phosphorylation (by WNK/SPAK) activate the NKCCs and inhibit the KCCs, leading to [Cl⁻]ᵢ increase and thus cellular regulatory volume increase (RVI). Protein phosphorylation and dephosphorylation is likely the key mechanism by which acute modulation of the cotransporters occurs; long-term modulation of the transporters is mediated by mechanisms that involve gene transcription and protein degradation.

This review will focus on the Na⁺ dependent chloride transporter NKCC1 and its involvement in Cl⁻ regulation in cells in both the nervous system and vasculature. NKCC1, which is encoded by the gene SLC12A1, symports 1Na⁺, 1K⁺, and 2Cl⁻ ions across the plasma membrane per transport cycle. In the case of secretory epithelia, NKCC1 transports ions across the basolateral membrane from the blood stream. Cl⁻ is then exported across the apical membrane by various Cl⁻ channels. NKCC1 is expressed early in mammalian development in neurons, glia, the spinal cord, peripheral nervous system (PNS), and cerebral vascular endothelial cells.

NKCC regulation of Cl⁻ in the mammalian brain and central nervous system

In the mammalian nervous system, the primary permeant anion is Cl⁻. Tight regulation of its flux in and out of cells is critical for a variety of neurophysiological processes. Intracellular Cl⁻ levels in neurons must be fine-tuned to ensure proper electrical output response to the amino acid neurotransmitter γ-aminobutyric acid (GABA).

GABA is the primary inhibitory neurotransmitter in the adult brain, gating electrical activity and serving as a balance against glutamatergic excitatory inputs. In neurons, GABA binds ligand-gated GABA_A receptors (GABA_AR), triggering conformational changes that facilitate the passive influx or efflux of Cl⁻ depending on the E₃Cl⁻. The [Cl⁻]ᵢ serves as an indicator of how a cell will respond to GABA; when [Cl⁻]ᵢ is low, such that E₃Cl⁻ is negative relative to the neuron membrane potential (Vₘ), activation of GABA_AR causes an influx of Cl⁻ and hyperpolarization of the neuron, but when [Cl⁻]ᵢ is high, and E₃Cl⁻ is positive compared to Vₘ, activation of GABA_AR results in an efflux of Cl⁻ and depolarization of the neuron.

Stage of development is a key determinant of the [Cl⁻]ᵢ of a neuron. During the postnatal period in rodents there is an as of yet undefined developmental switch that causes GABA to shift from being excitatory to inhibitory as the brain matures. Mature neurons in the central nervous system (CNS) maintain a low [Cl⁻]ᵢ. This results in E₃Cl⁻ being close to the neuron’s resting Vₘ and for GABA to elicit a hyperpolarizing effect on the neuron membrane potential by opening GABA_ARs and allowing influx of Cl⁻. The low levels of intracellular Cl⁻ in the mature CNS is primarily the result of higher expression levels of KCC2, which is only expressed in neurons in the CNS, and low levels of NKCC1. During development, on the other hand, there are low levels of KCC2 expression in the brain and relatively high levels of NKCC1, facilitating GABA depolarizing and excitatory responses. As a result of this expression pattern, immature CNS neurons have a high [Cl⁻]ᵢ (20–40 mM higher than mature neurons), with the electrochemical driving force of Cl⁻ out of the cell leading to a
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depolarizing Cl\(^{-}\) equilibrium potential (Table 1\(^{1}\)). The evolutionary advantage of the switch in NKCC1 expression remains an unanswered question in the field. Still, even minute changes in the \([\text{Cl}^{-}]_{i}\) of a neuron can drastically alter synaptic strength as well as the polarity of GABAergic neurotransmission.

Intracellular levels of Cl\(^{-}\) are also an important osmotic factor of cell volume, as is setting the direction of GABA\(_{A}\)R-mediated currents, since water flows in the same direction as the flux of ions\(^{7}\). Neurons, glia, and other cells in the brain utilize \([\text{Cl}^{-}]_{i}\) as a tool to defend against cell volume perturbations such as fluctuations in extracellular osmolality or intracellular solute or ion content that can compromise their structural integrity. Cl\(^{-}\) homeostasis in neurons is thus a balance between cell volume regulation and GABAergic signaling; imbalance of this homeostasis has been linked to human diseases where the robust activity of NKCC1 is thought to contribute to neonatal seizures\(^{1}\).

NKCC1 is also highly expressed in the mammalian choroid plexus, a specialized epithelium that plays an important role in the production and regulation of CSF. The choroid plexus is composed of a single layer of epithelial cells that forms a tight barrier resulting from the tight junction complexes between the cells, effectively separating the cerebrospinal fluid (CSF) from the blood\(^{12}\). The choroid plexus thus restricts the passage of molecules and ions into the CSF. Additionally, the choroid plexus lines the ventricles of the brain, and by doing so supplies neurons and glia with essential nutrients from the periphery, removes toxins from the CNS, and facilitates communication between the neuroendocrine system and the brain\(^{13}\). In contrast to NKCC1 expression on the basolateral membrane of secretory epithelia, NKCC1 is expressed on the apical membrane of the choroid plexus where it mediates Cl\(^{-}\) efflux due to the high Cl\(^{-}\) and Na\(^{+}\) concentrations in the epithelial cells of the choroid plexus (Table 1\(^{14}\)). However, others argue that NKCC1 causes an influx of ions in the choroid plexus epithelial cells, rather than an outward flow\(^{13}\). Because the integrity of the choroid plexus and the composition and volume homeostasis of the CSF is imperative for proper brain function, further characterization of NKCC1 function in the choroid plexus is warranted.

<table>
<thead>
<tr>
<th>Tissue:</th>
<th>Level of NKCC1 expression</th>
<th>Cell polarity</th>
<th>([\text{Cl}^{-}]_{i})</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Low</td>
<td>--</td>
<td>7(^{15})</td>
</tr>
<tr>
<td>Cells in the developing</td>
<td>High</td>
<td>--</td>
<td>30-50(^{11})</td>
</tr>
<tr>
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<td>Apical membrane of epi-</td>
<td>65(^{16})</td>
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<tr>
<td>Endolymp of inner ear</td>
<td>High</td>
<td>Basolateral membrane of</td>
<td>110(^{17})</td>
</tr>
<tr>
<td>Dorsal root ganglia</td>
<td>High</td>
<td>--</td>
<td>30-45(^{18})</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>High</td>
<td>--</td>
<td>30-51(^{19})</td>
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*Table 1.* NKCC1 expression and \([\text{Cl}^{-}]_{i}\) in various cell types.
NKCC regulation of Cl⁻ in the mammalian peripheral nervous system

In addition to regulating Cl⁻ levels in the developing CNS and choroid plexus, NKCC1 plays a key role in maintaining Cl⁻ homeostasis in the PNS. For example, NKCC1 is implicated in mammalian cochlear and vestibular function: SLC12A2 knockout mice are deaf and display head bobbing and circling behavior known as “shaker/waltzer” behavior²⁰²¹. These phenotypes are associated with pathologies in the chambers of the inner ear. The inner ear contains a specialized extracellular fluid known as the endolymph, which bathes the sensory organs of the inner ear²⁰. The endolymph contains high K⁺ and low Na⁺ levels, an atypical ion composition for an extracellular space that instead more closely resembles that of the intracellular milieu²¹. The endolymph is generated by marginal cells of the stria vascularis in the cochlea, as well as by dark cells in the vestibular system²².

Endolymph volume is largely regulated by secretion of K⁺ ions. Mutations in the genes encoding for proteins that regulate K⁺ endolymphatic homeostasis results in a loss of endocochlear potential, a reduction in endolymphatic volume, and sensorineural deafness due to hair cell degeneration²⁰. NKCC1 is a key contributor to K⁺ secretion in the endolymph. NKCC1 is expressed on the basolateral membrane of marginal cells²³ and transports K⁺ ions into the marginal cells from the intrastrial fluid, which is then channeled out from the cell into the endolymph by K⁺ channels (Table 1). Mice with complete loss of NKCC1 function demonstrate reduced endolymph volume and degeneration of hair cells²⁰. Interestingly, over-administration of loop diuretics in pediatric subjects can lead to reversible hearing loss²⁴²⁵. This ototoxicity is the result of decreased K⁺ secretion and loss of the endolymphatic potential²⁶.

NKCC1 and its regulation of intracellular Cl⁻ are important for sensory afferent signaling in the PNS. Unlike the CNS where NKCC1 undergoes a developmental downregulation from immature neurons to mature neurons, high NKCC1 expression persists in sensory afferent neurons throughout development and adulthood²⁷ (Table 1). This is the case for cranial ganglia such as the vestibular and spiral ganglia, as well as for dorsal root ganglia (DRG)²⁸. DRGs contain the cell soma of primary afferent sensory neurons, which modulate, gate, and transduce sensory information from the periphery to the spinal cord²⁹. Several classifications of DRG neurons exist, and they are characterized by the size of their cell bodies, and function. Type A DRG neurons are large and are coupled to touch, vibration, and proprioception³⁰, whereas type B neurons are smaller than their type A counterparts. Although smaller, type B neurons exceed the number of type A neurons with a ratio of 7:29 and are associated with the sensation of pain or nociception³¹.

Not only do DRG cell bodies function as metabolic stores for peripheral processes, but studies have shown that they are also heavily involved in signaling. DRG cells identify specific molecules and respond by producing other molecules in order to modulate the signaling processes³². Humans have 31 pairs of spinal nerves carrying autonomic and sensorimotor information located between the spinal cord and the periphery³³. The spinal nerves are composed of dorsal afferent sensory axons that become dorsal roots, as well as ventral efferent motor axons that become ventral roots. The spinal nerves emerge from the intervertebral neural foramina located between adjacent vertebrae and between the superior and inferior pedicles. The dorsal sensory root fibers travel laterally to connect their processes at a T-junction with their cell bodies, making up the DRG. Anatomically, the primary sensory nerve arises from the distal axons of the dorsal sensory root. The neuron’s peripheral receptive field is the start of the primary sensory neuron³⁴. The receptive field is the region in which a stimulus, such as touch, injury, or inflammation, initiates neuronal firing, and ends at the CNS. The primary afferent neurons (PANs) are the largest neurons in the human body, reaching up to 1.5 m in length³⁵.
As stated previously, NKCC1 expression is high in PANs in DRG to maintain a high [Cl−], (Table 1)28,18. This makes it possible for PANs to be consistently depolarized by GABA throughout adulthood36. GABAergic depolarization in PANs is mediated by Cl− efflux facilitated by GABAAR channels: the electrochemical driving force of Cl− is outward because ECl is positive compared to Vm37. The outward Cl− gradient is maintained throughout the entire cell surface of the PAN, including the cell soma in the DRGs, the central processes, as well as the peripheral processes37. The exact mechanism of the excitatory depolarizing effect of GABAARs remains unknown, as is why excitatory depolarization does not lead to shunting inhibition as seen in mature neurons of the CNS. However, the extent of our current knowledge on the depolarizing effect of GABA is that an increase in NKCC1 activity results in an increase in [Cl−], thereby increasing GABA-mediated depolarization above action potential threshold27 (Figure 1).

It is also thought that voltage-gated Ca2+ channel activation can lead to GABAergic depolarization by depolarizing the membrane enough to reach action potential threshold, resulting in a depolarization amplification effect38. Although Cl−-mediated GABA depolarization is the main depolarization mechanism in sensory afferents, a subset of DRG neurons involved in nociception depolarize via the Ca2+-dependent depolarization mechanism. Data demonstrate that NKCC1 mRNAs and protein are expressed in virtually all types of DRG neurons28. In addition to confirming that NKCC1 is the key player in maintaining [Cl−], above equilibrium, studies have revealed that there is a downward “drift” in [Cl−], at various postnatal ages in single DRG neurons, although [Cl−], remains higher than ECl27. However, a rapid transition in [Cl−], does not occur as observed in neurons of CNS. In fact, KCC2 transcripts and protein are absent in DRG neurons, suggesting that Cl− homeostasis cannot be attributed to KCC2. These findings suggest that Cl− influx is unopposed by KCC2-mediated outward Cl− transport and support the Cl−-mediated GABAergic depolarization model underlying PAD in the spinal cord. Nevertheless, the Cl− gradient in PANs plays a significant role at the peripheral endings by contributing to the rapid excitation of nociceptors by chemical mediators of pain that are released at the time of tissue damage69.

Similarly, at the central terminals, the Cl− gradient eventually gives rise to GABA-mediated PAD by spinal interneurons. PAD underlies presynaptic inhibition, a pervasive mechanism that is crucial in the gating and modulation of somatosensory information40. Particularly, PAD decreases the relative intensity of incoming action potentials resulting in reduced PAN neurotransmitter release. GABAAR antagonists reduce the effect of PAD41 suggesting that PAD is mediated by spinal interneuron GABA release. Supporting this hypothesis are data from behavioral tests conducted on NKCC1 knockout mice measuring pain. NKCC1 knockout mice exhibited a reduced response to noxious heat and touch-evoked pain42,43. To further confirm the association of NKCC1 and the pain pathway, mice were treated with the NKCC1 specific inhibitor bumetanide via intrathecal delivery44. Bumetanide was found to inhibit nocifensive behavior of formalin tests44. Furthermore, intracolonic capsaicin injections in mice stimulated a rapid and transient increase in spinal phosphorylated NKCC1, which is the activated state of the transporter, and persistent trafficking of NKCC1 to the cell surface45. In enhanced pain states, the enhancements of PAD might be necessary for inducing direct activation of spinal nociceptors instead of inhibiting incoming action potentials, a phenomenon known as dorsal root reflexes46. Taken together, these findings indicate that NKCC1 might be a fundamental regulator of inflammatory and tissue damage in the sensory afferent pain pathway.
**Figure 1. Depolarization effect of GABA in sensory afferents.** High expression of NKCC1 in dorsal root ganglia maintains a high $[\text{Cl}^-]_e$. The electrochemical driving force for $\text{Cl}^-$ causes GABA$\_R$ mediated $\text{Cl}^-$ efflux, leading to membrane depolarization.

**NKCC and Cl$^-$ regulation in smooth muscle cells**

Each major organ in the human body sends afferent fibers, the cell bodies of which reside in the DRGs, to the spinal cord to modulate autonomic function. Regulation of Cl$^-$ levels are both indirectly and directly vital for establishing the vascular tone in the smooth muscle cells (SMCs) of these organs.$^{47}$ Interestingly Cl$^-$ conductance through SMC membranes is much higher than that of skeletal and cardiac muscle cells.$^{48}$ This is of particular importance in muscle contraction, as SMCs do not generate action potentials spontaneously in the presence or absence of agonists but instead rely on an increase in Cl$^-$ conductance to stimulate muscle contraction (Table 1).

Contractile activity in SMCs in response to specific stimuli is initiated by an increase in Ca$^{2+}$, interaction between Ca$^{2+}$ and calmodulin, and ultimately phosphorylation of and thus stimulation of light chain myosin.$^{49}$ The main source of Ca$^{2+}$ for the tonic contractile activity of muscle is thought to come from extracellular stores channeled through L-type Ca$^{2+}$ channels, although Ca$^{2+}$ is additionally obtained from intracellular stores such as the sarcoplasmic reticulum.$^{47}$ L-type Ca$^{2+}$ channels are voltage operated Ca$^{2+}$ channels expressed at the plasma membrane that open in response to membrane depolarization. The activation of Cl$^-$ channels and/or transporters and changes in $[\text{Cl}^-]_e$ are a determining factor in tonic contraction as they regulate the activity of L-type Ca$^{2+}$ channels as well as contribute to membrane depolarization. Specifically, high $E_{\text{Cl}}$ in SMCs activate L-type Ca$^{2+}$ channels and blocks spontaneously generated action potentials in excitable SMCs.

The exact role of Cl$^-$ contribution to contraction and vascular tone in SMCs still remains unclear. However, several studies have revealed a potential contribution of NKCC1 to intracellular Cl$^-$ levels in maintaining vascular tone in SMCs.$^{50-51}$ One study showed that NKCC1 expression in rat aorta is stimulated by vasoconstrictors and inhibited by nitrovasodilators.$^{52}$ In addition, in the same study, when NKCC1 was inhibited with the loop diuretics bumetanide or furosemide the contraction of aorta and other large vessels as well as $[\text{Cl}^-]_e$ were decreased in vitro. Consistent with these findings, another study demonstrated NKCC1 upregulation in hypertension.$^{53}$ Further, mice that lack NKCC1 have been shown to have a reduction in SMC contraction.$^{54}$ Studies in which furosemide was administered to isolated resistance arteries found a reduction in the tone of these vessels as well as systemic vasodilation.$^{55,56}$ However, because furosemide can inhibit other transporters
and Cl channels, NKCC1 knockout mice and bumetanide administration in mice have also been utilized to examine the role of NKCC1 in vascular tone. Results from these studies showed an absence of vasoconstriction of SMCs in these mice, providing further evidence that the inhibition of NKCC1 can itself produce vasodilation and is likely the transporter responsible for the vasodilatory effect of furosemide.

An Undiagnosed Disease Program Case

Until recently, NKCC1 has not been linked to any inherited or non-inherited forms of human disease, possibly because of the potential for embryonic lethality should a mutation occur in the SLC12A2 gene. However, this lack of connection changed when an 8 year old patient with multi-organ failure was admitted to the Undiagnosed Diseases Program (UDP) at the NIH. In order to better understand the disease, the patient’s physicians initially ran several tests and identified an abnormal increase in glycogen content as well as mitochondrial DNA copy number in the patient’s muscle and liver cells. In addition to identifying what seemed to be a metabolic deficit, whole exome sequencing conducted on the patient’s genome revealed that the patient had several mutations in protein encoding genes, including a novel heterozygous mutation in NKCC1. Due to the widespread expression of NKCC1 and its importance in many cellular functions, the NKCC1 mutation was given the highest investigative priority.

This case was presented to our laboratory to further investigate the impact of this NKCC1 heterozygous mutation. We confirmed that the patient carries an 11 base pair deletion in the NKCC1 gene, leading to a frameshift and to the introduction of a stop codon that results in a COOH-terminal truncation of the protein. The mutant was called DFX for the last “intact” residue, aspartic residue (D), followed by the introduced residue, phenylalanine (F), and finally the stop codon (X). Rubidium tracer flux assays in Xenopus oocytes expressing the NKCC1-DFX mutation showed that the transporter was non-functional. Concomitantly, immunocytochemistry performed on HeLa cells transfected with mutant NKCC1-DFX and WT NKCC1 revealed that the cells transfected with mutated NKCC1 had a diffuse expression of GFP tagged NKCC-DFX in the cytoplasm compared to the stronger expression at the plasma membranes in cells expressing WT tdTomato tagged NKCC1. Western blot analysis uncovered an increase in dimerization between the wild-type and mutant transporter, indicating that the mutant protein might exert a dominant negative effect on the wild-type transporter. Two hypotheses to explain how NKCC-DFX might contribute to the observed multi-organ failure have been advanced: 1) disruption of proper afferent feedback mechanisms, as all tissues send afferent fibers back to the spinal cord; and 2) disruption of smooth muscle cell function which then can affect gastrointestinal organs, bladder, and vasculature.

Concluding remarks

NKCC1 is highly relevant for the function of two very different epithelia: the choroid plexus and the stria vascularis of the inner ear. In the choroid plexus, the cotransporter is located on the apical membrane where it might participate in CSF secretion or K+ reabsorption based on the level of intracellular Cl-. In the inner ear, the cotransporter participates in the formation of the K⁺-rich endolymph, which is important for hearing (cochlear cavity) and balance (vestibular cavity). This review discussed the need for regulation of intracellular Cl− in a variety of different cell types. In the CNS, NKCC1 undergoes a developmental downregulation of expression, which leads to differences in [Cl−]i in immature vs. mature neurons. This developmental switch is associated with a shift in GABA responses from depolarizing (excitatory) to hyperpolarizing (inhibitory). In the PNS, conversely, NKCC1 expression remains high throughout adulthood, which is important for maintaining high [Cl−]i levels for primary afferent depolarization, presynaptic inhibition, and the filtering of sensory noise. All major organs possess smooth muscle cells where NKCC1 maintains high [Cl−]i to facilitate the opening of voltage activated Ca2+ channels and SMC contraction. Taken together, the NKCC1 transporter is
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critical for the homeostatic operation of many physiological systems. Because loss of function of NKCC1 can lead to detrimental diseases, further understanding of how this transporter functions and how it is developmentally regulated is critical for developing novel therapeutics that may alleviate or prevent these diseases.

References


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Diverse Roles of GABA\textsubscript{A} Receptor Subunit Mutations in Epileptic Encephalopathies

Sarah Poliquin

Abstract

Epilepsy is the most common neurological disorder worldwide, with a large range in disease severity. The most severe forms, epileptic encephalopathies (EEs), are characterized by seizures that begin in infancy or early childhood and developmental delays that may leave the patient with life-long intellectual disability, in addition to other comorbidities. Approximately one-third of patients do not find a treatment that is effective in controlling their seizures. Thankfully, the explosion of genetic sequencing in recent years has been of tremendous help in identifying genetic mutations that contribute to epilepsy. Some of the genes most commonly associated with epilepsy are the components of \(\gamma\)-aminobutyric acid (GABA) receptors. GABA acts to inhibit neuronal firing, lowering the chance of a storm of abnormal electrical activity spreading throughout the brain and causing a seizure. Mutations in the receptor subunits \(GABRA1\), \(GABRB1-3\), and \(GABRG2\) have been recently linked with EEs. Progress is being made in understanding how specific mutations in these genes contribute to epilepsy. It appears that there may be several different mechanisms via which EE can result from mutations in GABR subunit genes, including disruption of GABR assembly, decreased function of GABRs, and impaired trafficking of GABRs to the cell surface. Optimal treatment of EEs will require a deeper understanding of these various mechanisms.

Keywords: epilepsy, GABA, GABA\textsubscript{A} receptor, endoplasmic reticulum, DNA sequencing

Epileptic Encephalopathies Have Diverse Genotypes and Phenotypes

Epilepsy is a broad category of brain dysfunctions that are characterized by seizures. Approximately 1% of the global population is affected, making epilepsy the most common neurological disorder. Epilepsy can be acquired through trauma or illness but often the cause is less clear, and these cases were originally denoted as idiopathic. Thanks to modern DNA sequencing technologies, many cases of idiopathic epilepsy have been shown to be the result of genetic mutations\textsuperscript{1}. Epilepsy phenotypes vary drastically, ranging from relatively mild conditions such as childhood absence seizures or febrile seizures to severe diseases such as Lennox-Gastaut syndrome (LGS) or Dravet syndrome (DS)\textsuperscript{2,3}. Many of the severe epilepsies are also characterized by intellectual disability (ID), which is sometimes worsened by seizure activity.

When the neurodevelopmental impairment is worse than would otherwise be expected based on the underlying cause, and this unexpected severity seems to be in part due to the epileptiform abnormalities, the condition is classified as an epileptic encephalopathy (EE)\textsuperscript{4}. The role of epileptiform electrical activity in possibly contributing to the ID can be difficult to determine, so in practice, if both severe seizures and ID are salient phenotypes, the patient will likely receive a diagnosis of EE even if it is not known how the seizure activity relates to the ID\textsuperscript{4}. The group of diseases classified as epileptic encephalopathies—e.g., infantile spasms/West syndrome (IS), Dravet syndrome/severe myoclonic epilepsy in infancy (DS/SMEI), Landau-Kleffner
(LKS), Lennox-Gastaut syndrome (LGS), early myoclonic encephalopathy (EME), and early infantile epileptic encephalopathy/Ohtahara syndrome (OS)—are quite heterogeneous \(^1\)\(^-\)\(^5\). The onset of these diseases ranges from early infancy for some up to five or more years of age for others, and there is also diversity in the type(s) of seizures present in a given disease, EEG patterns, comorbidities, and usual outcome \(^4\)\(^-\)\(^5\).

Just as heterogeneous as the disease phenotypes are the associated genotypes: as of 2016, over 70 different genes have been associated with EEs (although a true causal role remains to be found for many), but this only accounts for 20-25% of idiopathic EE cases \(^1\). Once a gene has been confirmed to cause epilepsy when mutated, more questions develop—why is it that two different mutations in the same gene can cause very different phenotypes? For example, one missense mutation in GABRB3 causes childhood absence epilepsy (a mild epilepsy) while a similar missense mutation causes LGS \(^6\)\(^-\)\(^7\). Conversely, two patients diagnosed with the same EE and displaying the same symptoms may have mutations in different genes unrelated in function, like GABRG2 and CHD2, a DNA binding protein, both of which are associated with DS \(^8\). Many of the EE-associated genes identified thus far code for ion channels, including both ligand-gated ion channels such as GRIN2A (a glutamate receptor subunit) and voltage-gated ion channels like SCN1A (a sodium channel), but not all \(^9\). Further examples of the variety of genes linked to EE include DNM1, a cell structure protein; PLCB1, a phospholipase; and STXBP1, a regulator of vesicle release \(^9\).

**The GABA\(_{A}\) Receptor Acts as an Inhibitor in the Central Nervous System**

In regards to epilepsy pathology, one of the most important ion channels is the GABA\(_{A}\) receptor (GABR). Long before connections between epilepsy and any gene had been uncovered, GABA signaling had been associated with epilepsy, due to knowledge that most GABA signaling is inhibitory and to discoveries that several antiepileptic drugs (AEDs) bind to GABA receptors \(^6\)\(^-\)\(^9\). The neurotransmitter GABA has two classes of receptors, the ionotropic GABA\(_{A}\) receptor and the metabotropic GABA\(_{B}\) receptor. In the developed brain, both act as inhibitory receptors—activation of the receptor by GABA results in hyperpolarization of the neuron, thus making the neuron less likely to reach the action potential threshold when it next receives an excitatory signal.

The type A receptor mediates the majority of inhibitory neurotransmission in the adult brain by hyperpolarizing a neuron with an influx of negative chloride ions in response to the binding of two GABA molecules \(^2\)\(^,\)\(^3\)\(^,\)\(^11\). The receptor is a heteropentamer consisting of five subunits arranged in a circle, with the electrically conducting pore in the middle, through which chloride ions flow after the pore opens \(^2\)\(^,\)\(^3\)\(^,\)\(^11\)\(^,\)\(^12\). All subunits are similar in structure—they consist of a large extracellular N-terminal domain and four transmembrane domains, with the second one lining the central pore \(^13\)\(^,\)\(^14\). The subunit composition of a functional receptor is highly variable as there are a total of nineteen different subunits: α1-6 (GABRA1-6), β1-3 (GABRB1-3), γ1-3 (GABRG1-3), δ (GABRD), ε (GABRE), θ (GABRQ), π (GABRP), and ρ1-3 (GABRR1-3) \(^2\)\(^,\)\(^3\)\(^,\)\(^11\)\(^,\)\(^12\). The ε, θ, π, and ρ subunits are rarely used; most GABRs contain two α and two β subunits and either a γ or δ subunit, with the most common arrangement being α1β2γ \(^2\)\(^,\)\(^3\)\(^,\)\(^12\). Of the nineteen subunits, seven have been implicated in epilepsy—GABRA1/6, GABRB1-3, GABRG2, and GABRD—so far \(^2\)\(^,\)\(^3\)\(^,\)\(^7\)\(^,\)\(^11\)\(^,\)\(^15\). However, like with many other epileptogenic genes, there is a wide variety of the phenotypes associated with mutations in these genes, and GABRA6 and GABRD have only been associated with milder epilepsies at the time of writing, leaving GABRA1, GABRB1-3, and GABRG2 as subunit genes identified as causes of EEs. Despite the clear role of GABA in neuronal inhibition, the precise mechanisms behind these genetic epilepsies are unclear, as there often appears to be more at play than loss of function, such protein aggregation, ER stress, and neurodegeneration \(^2\)\(^-\)\(^3\).
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Mutations in GABRA A Can Cause Epileptic Encephalopathies

The gene encoding the α1 subunit, GABRA1, was the first gene convincingly shown to be a likely cause of idiopathic epilepsy, in a 2002 study on a family with juvenile myoclonic epilepsy\textsuperscript{16}. A connection between EE and GABRA1 was made in 2013, thanks to the increased availability of whole exome sequencing for a large number of subjects\textsuperscript{7}. The international group Epilepsy and Pharmacogenetics (Epi4K) Consortium analyzed a large cohort with EEs and identified a missense mutation in GABRA1 in a patient with IS, as well as finding many other novel mutations of several genes in other patients\textsuperscript{7}. The following year, Carvill et al screened 67 DS patients that were negative for SCN1A mutations (SCN1A mutations account for \textasciitilde75\% of DS patients) and found four patients (6\%) with de novo heterozygous missense mutations in GABRA1: two with R112Q, one with G251S, and one with K306T\textsuperscript{17}. Johannessen et al identified a further fifteen patients in 2016 with de novo missense mutations likely responsible for the patients’ EEs: eight with DS or DS-like symptoms (S76R, R112Q x2, L146M, R214H, G251S, T292I, K306T), four with early-onset epileptic encephalopathy (S76R, R214H, T289P, T289A), one with severe myoclonic-astatic epilepsy-like symptoms (K306T), and two with mild EE (N115D, G251D)\textsuperscript{15}.

Because these mutations have only been discovered in the past few years, little research has been done to elucidate the biochemical, cellular, and network effects. However, Carvill et al and Johannessen et al both include some preliminary electrophysiology data for some of the GABRA1 mutations they discovered. They transfected Xenopus laevis oocytes with cDNA for human GABRA1 in either wildtype or mutated form, as well as GABRB2 and GABRG2 in order to allow the formation of a functional receptor\textsuperscript{17}. Upon application of GABA, oocytes expressing mutant α1 displayed a 40-70\% reduction in peak current compared to oocytes transfected with only wildtype cDNA\textsuperscript{17}. Additionally, both groups observed shifts in the dose-response curve indicative of a decrease in sensitivity to GABA compared to wildtype\textsuperscript{17}. It is important to note that both studies used homozygous mutated cDNA; when expressed heterozygously with wildtype GABRA1, the current was reduced only about half as much as in the mutant homozygous condition, suggesting that these mutations are acting in a loss-of-function manner\textsuperscript{15}.

Also in 2016, Kodera et al sequenced several hundred patients with early-onset EE (EOEE), looking for more mutations in GABRA1\textsuperscript{12}. They found six patients with de novo missense mutations in GABRA1, three of which have IS (M263T, M263I, P260L), one of which was initially diagnosed with OS that progressed to IS (P260L), one with an unspecified EOEE (V287L), and, interestingly, a patient with a mutation that had previously been associated only with DS (R112E)\textsuperscript{12}. This patient had a seizure profile indicative of DS but showed no motor developmental delay and only mild ID that was not worsening; this highlights the poorly-understood and vast heterogeneity of epilepsies resulting from mutations in the same gene\textsuperscript{12}. To gain some understanding of the biochemical nature of these mutations, Kodera et al expressed three of their newly-identified mutations (R112E, P260L, and M263T) with FLAG tags in HEK293 cells to determine the cellular locations of the proteins, and found that the mutated protein localized at the cell periphery, as does wildtype α1, suggesting that these three mutant subunits may assemble into full GABA\textsubscript{A} receptors and decrease receptor function\textsuperscript{12}.

Mutations in GABRB1-3 Can Cause Epileptic Encephalopathies

The β3 gene GABRB3 was one of the first GABR subunits to be associated with epilepsy—in 1999, linkage analysis suggested that GABRB3 may contribute to the etiology of childhood absence epilepsy (CAE), although the association was not yet strong\textsuperscript{8}. In regards to EE, it was not until the 2013 sequencing study by the Epilepsy Phenome/Genome Project that a GABR β subunit was implicated\textsuperscript{7}. That study identified four novel de novo missense mutations in GABRB3 associated with EE, as well as one novel de novo missense mutation in GABRB1\textsuperscript{7}. Three of the patients have LGS (D120N, E180G, Y302C) and, at the
time of the study, two—including the child with the GABRB1 mutation—have IS that may later progress to LGS (GABRB3(N110D) and GABRB1(F246S))

After these mutations were identified, Janve et al extensively investigated the cellular effect of these mutations by transfecting HEK293 cells with cDNA for wildtype or mutant β subunits, as well as wildtype GABRA1 and GABRG2 cDNA so that a full pentameric receptor could be formed\textsuperscript{11}. The total and surface expression of some of the mutant β subunits (β1(F246S), β3(N110D), and β3(Y302C)) varied in comparison to wildtype β1/3, suggesting that the trafficking of the mutant subunits may be abnormal\textsuperscript{11}. However, in the homozygous condition, none of the mutations had any change on the total levels of α1 or β2 compared to the levels seen when wildtype GABRB1/3 were used, nor was surface expression affected, indicating that the abnormal trafficking of the mutant subunits does not impair assembly or trafficking of the full pentamer\textsuperscript{11}. Electrophysiology revealed that β3(D120N) showed decreased potency of GABA but the efficacy was unaffected, while the E180G and Y302C mutants displayed decreased efficacy\textsuperscript{11}. Additionally, channel opening/deactivation kinetics were altered in all the mutants\textsuperscript{11}.

Shortly after the publication of the work of Janve et al, a separate lab reported a second patient with a mutation in GABRB1 (T287I) that presents with intractable epilepsy and severe developmental delay\textsuperscript{18}. The biochemistry of that mutation has not yet been characterized although the position at which the mutation occurs is highly conserved, as it is part of the pore-lining second transmembrane domain\textsuperscript{18}. Papandreou et al report the same mutation in GABRB3 in a patient with similar symptoms, which, given the high degree of homology between the genes, is unsurprising\textsuperscript{13}.

The rate of mutation discovery picked up in 2016, starting with the Epi4K Consortium identifying seven EE patients with missense mutations in GABRB3\textsuperscript{19}. Another group reports a patient with EOEE and a de novo missense mutation in GABRB3 (Y302C) and, in a subsequent publication, additional patients with mutations in GABRB3\textsuperscript{19,20}. Twenty-two epileptic patients displayed a range of phenotypes, from mild febrile seizures to DS\textsuperscript{20}. The majority of the mutations are missense point mutations but there is also a duplication of exons 1-9 that resulted in IS\textsuperscript{20}. Duplications of GABRB3 have been found elsewhere to contribute to EE\textsuperscript{21}. Brief testing of the functional consequences of some of the newly identified mutations showed reduced current amplitudes in Xenopus oocytes\textsuperscript{20}. Remarkably, some of the patients with EE did not acquire their mutation de novo but rather inherited it, which is not seen in any of the other cases of GABR-related EE, suggesting that the GABRB3 mutation carried is but one of many factors that can contribute to the development of EE\textsuperscript{20}.

The first case of an association between GABRB2 and EE was reported in 2017 by Ishii et al, and only one other case of GABRB2 and epilepsy has been reported in the literature, in which a patient with GEFS\textsuperscript{+} has the mutation M797T\textsuperscript{22,23}. The EE patient described by Ishii et al presents with early myoclonic encephalopathy (EME), one of the most severe EEs\textsuperscript{22}. Genetic sequencing revealed a novel missense mutation in GABRB2, T287P\textsuperscript{22}. To investigate the effects of the mutation, GABRA1 and GABRG2 cDNA were transfected into HEK293 cells, as well as wildtype GABRB2 or mutant GABRB2(T287P) or a heterozygous mix\textsuperscript{22}. In both the homozygous mutant and heterozygous condition, total and surface expression of β2 was decreased relative to the wildtype condition, with an approximate 2-fold difference between homozygous and heterozygous conditions\textsuperscript{22}. The mutant proteins stayed near the nucleus (presumably in the ER) while wildtype β2 trafficked to the surface\textsuperscript{22}. Interestingly, when cells were co-stained for α1 and γ2, it was found that γ2 colocalized with the mutant β2, suggesting that the mutation affects the assembly of the full receptor\textsuperscript{22}. When patch-clamp recordings were taken, the current from cells homozygously transfected with GABRB2(T287P) was shown to have been nearly eliminated, decreased 96.4% from the wildtype peak amplitude\textsuperscript{22}. As surface expression was
only reduced 66%, the mutant is likely disrupting the functionality of the channel in addition to interfering with trafficking/assembly\textsuperscript{22}.

**Mutations in GABRB1-3 Can Cause Epileptic Encephalopathies**

The first study demonstrating GABRG2 as an epileptogenic gene came in 2001 and found a missense mutation carried by affected members of a large family with a history of GEFS\textsuperscript{+10}. The year following that publication, GABRG2 was linked to EE when a patient with DS was found to have a Q390X (formerly denoted Q351X) truncation mutation\textsuperscript{24}. The biochemical findings here were rather exciting: the mutation prevented the GABA\(_\lambda\) receptor from assembling, even though \(\alpha_1\) and \(\beta_2\) can form functional receptors without a third subunit, at least in vitro\textsuperscript{24}. Stimulation of oocytes transfected with the mutant resulted in no current at all, indicating that there were no functional receptors, as a strong current was observed in the wildtype-only oocytes\textsuperscript{24}. A green fluorescent protein (GFP) tag was added to both forms of γ2, which showed that while wildtype γ2 can be found in the intracellular compartment as well as the membrane, γ2(Q390X) was only found in the intracellular compartment, colocalized with ER markers\textsuperscript{24}. The authors propose that the mutant forms full pentameric channels, but the lack of the transmembrane-targeting signal at the C-terminus prevents γ2(Q390X) from leaving the ER\textsuperscript{24}. The \(\alpha\) and \(\beta\) subunits that have assembled with the mutant will therefore also be trapped in the ER, causing a reduction of functional receptors at the membrane\textsuperscript{24}.

Kang et al further investigated GABRG2(Q390X) extensively. They created transgenic heterozygous Gabrg2 knock out mice (\(\text{Gabrg}^{2+/-}\)) and mice with Gabrg2(Q390X) heterozygously knocked in (\(\text{Gabrg}^{2+/\text{Q390X}}\))\textsuperscript{25}. The Gabrg2\(^{2+/-}\) mice do not have a severe epilepsy phenotype, displaying only brief absence seizures in a seizure-prone genetic background, which is in stark contrast to the severe tonic-clonic seizures seen in the Gabrg2\(^{2+/\text{Q390X}}\) mice, even in a seizure-resistant genetic background\textsuperscript{25,26}. Because the Gabrg2\(^{2+/-}\) mice have only a mild phenotype that does not mimic the human phenotype of DS, haploinsufficiency of the truncated protein does not seem to be the cause of the disease\textsuperscript{25,26}. Rather, the fact that Gabrg2\(^{2+/\text{Q390X}}\) mice better match DS suggests that the truncated protein itself is directly contributing to the pathology in a dominant-negative manner\textsuperscript{25,26}. Biochemical analysis of the mice revealed that translated γ2(Q390X) protein quickly forms stable aggregates with itself and with \(\alpha_1\), creating a decrease of full receptors due to lack of available \(\alpha_1\)\textsuperscript{25-27}. The γ2(Q390X) aggregates were detectable in neonatal mice and continued to accumulate as the mice aged\textsuperscript{25}. In fact, older mice had elevated levels of activated caspase 3 and TUNEL, two markers of neuronal death, due to ER stress and the accumulation of γ2(Q390X)\textsuperscript{25}.

There is similar pathology in another GABRG2 truncation mutation, Q40X, found by Kanaumi et al in two twins with DS\textsuperscript{28}. Like γ2(Q390X), γ2(Q40X) and a portion of the total \(\alpha_1\) form granules in the cytosol rather than trafficking to the membrane, perhaps becoming trapped in the ER\textsuperscript{28}. When tested in oocytes, the heterozygous GABRG2(Q40X) condition decreased current amplitude further from the wildtype condition than the homozygous mutant condition did, indicating a dominant-negative effect of the mutation\textsuperscript{29}.

In 2017, Shen et al published a study in which they identified six novel de novo GABRG2 mutations in eight patients with EEs\textsuperscript{30}. Using transfected HEK293 cells, they analyzed the effect of the mutations on GABA-evoked current and found that all six reduced GABAergic current, but to differing degrees, depending on the protein domain in which the mutation was located\textsuperscript{30}. To see the effects of the mutations on γ2 biogenesis, they analyzed total levels of the protein and found that for four of the mutants, the amount of protein was similar to wildtype γ2, although there was decreased surface expression\textsuperscript{30}. The extent of the decrease in surface expression for each mutation was similar to the decrease of current, suggesting that the primary effect of these mutations is to prevent GABR assembly and surface trafficking\textsuperscript{30}. For the other two mutants (I107T and P282S), however, there was a significant increase in the total amount of protein compared to wildtype,
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despite decreased surface expression, which suggests that these variants are more stable than the others. Accor-
ding to co-staining experiments, I107T and P282S had increased intracellular protein that colocalized
with an ER marker; this suggests that the pathophysiology of these two mutations may be similar to that of
Q390X.

Conclusions

In the past twenty years, the field of idiopathic epilepsies has been revolutionized, in large part because of the
development of next-generation sequencing technology and its increasing affordability. The idiopathic epil-
epsies are now called genetic epilepsies, as many diseases have been shown to have strong genetic contribu-
tors. There is much crossover between genes causing severe EEs or mild CAE, so more discoveries in one dis-
ease will likely benefit another. The GABA\(_A\) receptor subunits especially span the spectrum of phenotypic se-
verity, although this review focused on the severe phenotypes. All but one of the subunits discussed was first
identified as a candidate epilepsy gene from investigating the milder epilepsies. Despite the advancements in
finding causes of patients’ epilepsy, the mechanisms of many mutations remain almost entirely unknown.
For some mutations, such as GABRG2(Q390X), what little is known points away from simple loss of func-
tion/haploinsufficiency and towards a more interesting—but also more complicated—mechanism involving
toxic gain of function. Identifying novel mutations is undoubtedly beneficial, but a thorough understanding
of each one is also necessary to bring maximum benefit to patients and their families.

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Potential mechanisms of parvalbumin inhibitory interneuron vulnerability in injury and disease states

Jacob Ruden

Abstract

Changes to parvalbumin-positive inhibitory interneuron structure and function have been reported in many injury and disease states, but the reasons why parvalbumin interneurons are especially vulnerable are not fully understood. In this review, I summarize what we know about parvalbumin interneuron function and parvalbumin interneuron-related changes in schizophrenia and Alzheimer's disease. Then, I explore aspects of parvalbumin interneuron biology that likely contribute to their vulnerability in disease and injury. The large demand for energy of these interneurons as well as their calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and N-methyl-D-aspartate (NMDA) receptor complement, are all likely to contribute to their vulnerability. The potential mechanisms by which these aspects of parvalbumin interneuron biology lead to the vulnerability of these interneurons are explored.

Keywords: parvalbumin, interneuron, GABA, calcium, vulnerability, energy, AMPA, NMDA, schizophrenia, Alzheimer's disease

Parvalbumin interneuron function

Parvalbumin interneurons are GABAergic interneurons that express a calcium-binding protein named parvalbumin ("small albumin"). Parvalbumin interneurons are also referred to as fast-spiking interneurons, because they are defined by their fast-spiking action potential phenotype. These interneurons are also defined by their parvalbumin immunoreactivity, although a parvalbumin interneuron can lose expression of parvalbumin and still exist as a formerly parvalbumin-positive interneuron. Parvalbumin interneurons have many unique qualities. They receive the greatest excitatory input of any population of inhibitory neurons in cortex. Parvalbumin interneurons have roles in both feedforward and feedback inhibition, the regulation of the magnitude of sensory responses, and the regulation of learning and plasticity. There are reports of an association between parvalbumin interneuron networks and plasticity early in life as well as in adult mice. Less is known about the protein that is the namesake of fast-spiking interneurons. Parvalbumin protein is reported to modulate short-term synaptic plasticity, quickly reduce presynaptic calcium, and prevent cumulative facilitation, but other functions of this calcium-binding protein have yet to be elucidated. Parvalbumin interneurons help to create and maintain gamma oscillations, which are high-frequency patterns of brain activity (defined as anywhere from 20-100 Hz depending on the author). Stimulation of parvalbumin interneurons leads to an increase in gamma oscillations, while inhibition of parvalbumin interneurons reduces gamma oscillation power. Additionally, a computational model demonstrates that networks with parvalbumin-deficient GABA synapses are likely to exhibit decreased gamma oscillations. Gamma oscillation power likely has a functional importance. For example, working memory load correlates with gamma oscillations in humans.
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Parvalbumin interneurons in injury and disease states

Parvalbumin interneuron-related changes have been reported in a variety of injury and disease states\textsuperscript{15}. Below is a summary of these changes in two diseases, schizophrenia and Alzheimer’s disease.

Schizophrenia. There are extensive accounts in the literature of parvalbumin interneuron-related changes in individuals with schizophrenia and in various animal models that recapitulate aspects of the disease. Humans with schizophrenia have reductions in the level of parvalbumin mRNA in the hippocampus and layer 4 of the dorsolateral prefrontal cortex, as well as reductions in the number of parvalbumin-positive interneurons in the hippocampus, caudal entorhinal cortex, and parasubiculum\textsuperscript{16-19}. Additionally, individuals with schizophrenia likely have reductions in the density of excitatory synapses selectively on parvalbumin-positive interneurons\textsuperscript{20}. Alterations in gamma oscillations during a working memory task in patients with schizophrenia have also been reported\textsuperscript{21}. Animal model findings replicate the reduction of parvalbumin-positive interneurons in the hippocampus\textsuperscript{22}. One area of debate is whether the interneurons themselves are lost or whether they simply lose expression of parvalbumin. Some human and animal model data suggest that formerly parvalbumin-positive interneurons are still present but no longer express parvalbumin\textsuperscript{23,24}.

Alzheimer’s disease. There are also reports of fast-spiking interneuron-related changes in Alzheimer’s disease. Mouse models with amyloid plaques, a hallmark pathology seen in Alzheimer’s disease, were found to have reduced gamma power that, in one model, precedes cognitive impairment or amyloid plaque formation\textsuperscript{25,26}. However, when hippocampal parvalbumin interneurons artificially produced gamma oscillations through the use of optogenetics in one of the mouse models, amyloid plaques were reduced\textsuperscript{25}. Separately, a voltage-gated sodium channel (Na.1.1) that is largely found at the axons of parvalbumin interneurons and that colocalizes with parvalbumin was found to be decreased in a mouse model with amyloid pathology and in Alzheimer’s disease patients compared with controls\textsuperscript{27-29}. When the levels of this voltage-gated sodium channel were restored in the mouse model with amyloid pathology, gamma oscillations increased, while memory deficits and premature death decreased\textsuperscript{29}.

There are conflicting reports of changes to parvalbumin interneurons themselves in Alzheimer’s disease. For example, decreases in the number of parvalbumin-positive interneurons in the hippocampus were observed in one mouse model of Alzheimer’s disease compared to non-transgenic mice and in another mouse model compared to younger pre-symptomatic transgenic mice\textsuperscript{30,31}. In a different mouse model, however, an increase in hippocampal parvalbumin immunoreactivity was observed relative to non-transgenic mice\textsuperscript{32}. In yet another mouse model, no change in the number of hippocampal parvalbumin-positive interneurons was observed compared to non-transgenic mice\textsuperscript{33}. The contradictions in the literature may be explained by the different mouse models used in each study. In humans, no change in parvalbumin-positive interneuron number density in frontal cortex was reported in brains from Alzheimer’s disease patients compared with control brains, a decrease in parvalbumin-positive interneurons was reported in the dentate gyrus in brains from Alzheimer’s disease patients compared with control brains, and an increase in parvalbumin-positive interneurons was reported in the piriform cortex in brains from Alzheimer’s disease patients compared with control brains\textsuperscript{31,34,35}. The different findings reported in each study are likely due to the different brain regions examined. In fact, it would be surprising to find that parvalbumin interneuron-related changes in response to injury or disease are uniform across every brain region containing fast-spiking interneurons, since a parvalbumin interneuron in prefrontal cortex does not necessarily have the same function as a parvalbumin interneuron in hippocampus.
Parvalbumin interneuron vulnerability

Since parvalbumin interneuron-related changes have been reported in such a wide variety of injury and disease states, it is reasonable to consider why this subtype of interneurons is especially vulnerable. There are many potential reasons that could explain why parvalbumin interneurons are particularly vulnerable, but, for the purposes of this review, I will focus on the large demands for energy of parvalbumin interneurons, the presence of calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on parvalbumin interneurons, and the N-methyl-D-aspartate (NMDA) receptor complement of parvalbumin interneurons. Note that the presence of parvalbumin protein itself might contribute to the vulnerability of parvalbumin interneurons, but, currently, not enough is known about parvalbumin and its functions to assess its role, if any.

Parvalbumin interneurons and their energy requirements. Parvalbumin interneurons are known to have high metabolic activity. It is unsurprising that these interneurons would have high metabolic activity, as this activity would reflect the characteristic fast-spiking quality of parvalbumin interneurons. In line with this view, there have been suggestions in the literature that parvalbumin interneurons have an especially high demand for ATP. Evidence in support of the high metabolic activity of this neuronal subtype include a large presence of cytochrome c and a large presence of cytochrome c oxidase (complex IV) in parvalbumin interneurons. Cytochrome c is required for the induction of apoptosis, programmed cell death. Thus, a high amount of cytochrome c in parvalbumin interneurons could mean that apoptosis is more likely to occur in this population of neurons than in other cell types. The high metabolic activity of parvalbumin interneurons could be required to support specific characteristics unique to this interneuron subtype. In the CA1 of rat hippocampus, parvalbumin-positive interneurons were found to have larger dendritic trees and thicker dendrites than two other classes of interneurons, calbindin-positive interneurons and calretinin-positive interneurons. In the same study, parvalbumin-positive interneurons had a higher density of inputs and a larger number of total excitatory and inhibitory synapses than calbindin- and calretinin-positive interneurons. Parvalbumin interneurons were also found to have a higher ratio of inhibitory to excitatory inputs than calretinin- or calbindin-positive neurons. Additionally, parvalbumin interneurons have a large number of mitochondria. Parvalbumin interneurons likely have a large number of mitochondria because the presence of many high-functioning mitochondria were shown to be required for gamma oscillations. In the same study, it was also shown that the amount of oxygen consumed during gamma oscillations is approximately equivalent to the amount of oxygen consumed during a seizure, and another study reported that the relative rate of oxygen consumption was significantly higher during hippocampal gamma oscillations than during the absence of action potential spiking or during spontaneous network activity.

High metabolic activity and a large demand for ATP are two qualities that could lead to parvalbumin interneurons being selectively susceptible to losing mitochondrial membrane potential (a proxy for a decrease in mitochondrial function) faster than other neurons and susceptible to the metabolic stress and oxidative stress that accompanies injury and disease states. There is good evidence supporting the notion that parvalbumin interneurons are selectively susceptible to oxidative stress. In the anterior cingulate cortices of mouse and rat models that recapitulate aspects of various disorders, higher oxidative stress consistently correlated with decreased parvalbumin-positive interneurons. Notably, in animal models where no change in oxidative stress was observed relative to controls, no change in parvalbumin-positive interneuron number was observed. Cause-and-effect could not be teased apart in that study. However, another study in mice found that the presence of oxidative stress caused a decrease in parvalbumin-positive interneurons in the hippocampus, as well as a reduction in gamma oscillations. Additionally, a study in rats found that the presence of oxidative stress caused a selective decrease in parvalbumin immunostaining in the anterior cingulate cortex.
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Parvalbumin interneurons and calcium-permeable AMPA receptors. Parvalbumin interneurons are known to express calcium-permeable AMPA receptors. The majority of hippocampal neurons expressing calcium-permeable AMPA receptors are parvalbumin interneurons, and calcium-permeable AMPA receptors are present in the dendrites of parvalbumin interneurons. In other cell types, NMDA receptors are the primary source of dendritic calcium, while in parvalbumin interneurons, dendritic calcium comes from both NMDA receptors and calcium-permeable AMPA receptors. The presence of calcium-permeable AMPA receptors are crucial for the function of parvalbumin interneurons. These receptors are required for long-term potentiation in a population of putative parvalbumin interneurons, and excitatory post-synaptic currents in parvalbumin-positive interneurons were reduced when a calcium-permeable AMPA receptor blocker was added. Parvalbumin interneurons have faster calcium influx kinetics than other cell types in part because of the fast calcium influx through calcium-permeable AMPA receptors.

The presence of calcium-permeable AMPA receptors in parvalbumin interneurons likely contributes to the vulnerability of these neurons. Rapid calcium influx through calcium-permeable AMPA receptors can cause calcium to enter mitochondria. The presence of calcium in mitochondria is known to generate damaging reactive oxygen species and to open a mitochondrial membrane conduction channel known as the permeability transition pore. Opening of the permeability transition pore can lead to disruption of mitochondria, release of cytochrome c, and ultimately result in apoptotic cell death. Mitochondrial disruption would be especially damaging to parvalbumin interneurons due to their high number of and high utilization of mitochondria. Slower but consistent calcium influx through calcium-permeable AMPA receptors can also be damaging, and it can result in the generation of nitric oxide. Nitric oxide, in turn, activates Poly(ADP-ribose) polymerase 1 (PARP-1). PARP-1 over-activation can lead to the release of apoptosis-inducing factor (AIF) via a nuclear signal that proliferates to mitochondria. As its name suggests, AIF ultimately induces apoptosis.

Calcium-permeable AMPA receptors are also highly permeable to zinc, and there is evidence suggesting that zinc enters neurons primarily through these receptors. Like calcium influx, zinc influx into neurons leads to the generation of nitric oxide and subsequent activation of PARP-1, ultimately resulting in cell death. Zinc is more potent than calcium in its ability to disrupt mitochondrial function. For example, unlike calcium, the entrance of zinc through calcium-permeable AMPA receptors can lead to a long-lasting production of the reactive oxygen species superoxide in mitochondria. Additionally, a higher absolute concentration of calcium influx compared to zinc influx is required to induce a similar increase in cytochrome c and AIF. Consistent with the notion that the presence of calcium-permeable AMPA receptors contributes to the vulnerability of parvalbumin interneurons, less cortical neurons die in an in vitro model that resembles traumatic brain injury when calcium-permeable AMPA receptors are selectively blocked, less hippocampal pyramidal neurons are lost in a mouse hippocampal slice model of oxygen-glucose deprivation in the presence of a calcium-permeable AMPA receptor blocker, and less retinal ganglion cells die in a rat model of glaucoma when these receptors are selectively blocked.

Parvalbumin interneurons and their NMDA receptor complement. The majority of parvalbumin interneurons in various brain regions of humans and monkeys have been shown to express NMDA receptors. Note that NMDA receptors are not universally expressed, as NMDA receptors were not found in the vast majority of calretinin-positive interneurons in various brain regions of monkeys and humans. NMDA receptors are heterotetramers that consist of at least one GluN1 (formerly known as NR1) subunit and at least one GluN2 (formerly known as NR2) subunit. There are four varieties of GluN2 subunits, GluN2A-GluN2D (NR2A-NR2D). The ratio of GluN2A-containing NMDA receptors to GluN2B-containing NMDA receptors was...
found to be five times higher in cultured parvalbumin interneurons than in pyramidal neurons, and GluN2A-containing NMDA receptor activity was determined to be critical for the preservation of parvalbumin immunoreactivity in cultured parvalbumin interneurons\textsuperscript{64}.

The dependence of parvalbumin interneurons on NMDA receptor function likely contributes to the vulnerability of these interneurons in response to conditions where NMDA receptors are not functioning properly. In schizophrenia, for example, NMDA receptors are believed to be hypo-functioning, and the inhibition of NMDA receptors recapitulates many of the symptoms of schizophrenia\textsuperscript{48,65}. Working memory is notably impaired in schizophrenia and in other diseases. Recent evidence from a study of rats suggests that working memory is dependent on GluN2A-containing NMDA receptors in the prefrontal cortex\textsuperscript{66}. In another study of rats, administration of a GluN2A-selective NMDA receptor antagonist resulted in an abnormal increase in gamma power, while administration of NMDA receptor antagonists that are selective for other subunits resulted in little-to-no change in gamma power\textsuperscript{67}. In a study of human brains from patients with schizophrenia and from normal controls, the brains from the individuals with schizophrenia had a lower density of GluN2A-expressing parvalbumin interneurons in layers 3 and 4 of prefrontal cortex compared to the brains from the control group\textsuperscript{68}. When this evidence is combined with the previously discussed finding that gamma oscillations correlate with working memory load in humans, it is likely that the decreases in working memory that are observed in diseases such as schizophrenia are in part mediated by the decrease in glutamatergic inputs through GluN2A-containing NMDA receptors onto parvalbumin interneurons.

\textbf{Figure 1: Contributions to parvalbumin interneuron vulnerability to injury and disease.}
Conclusion

I have presented evidence from three features of parvalbumin interneuron biology that may contribute to the vulnerability of these interneurons. The contributions of these aspects of fast-spiking interneuron biology are summarized in Figure 1. It should be emphasized that the contributions to their vulnerability in disease and injury are not occurring in isolation. For example, calcium could enter the mitochondria of parvalbumin interneurons via their calcium-permeable AMPA receptors. While the presence of calcium in mitochondria can be damaging in any situation, calcium in the mitochondria of parvalbumin interneurons would be especially damaging due to their high number of and high reliance on functioning mitochondria. It also should be noted that in no way are the facets of parvalbumin interneuron biology that I review here an exhaustive list of why parvalbumin interneurons are so vulnerable. For example, as discussed earlier, it is very possible that parvalbumin protein that defines these interneurons contributes to their vulnerability, but too little is currently known about the protein. Knowledge of what factors contribute to parvalbumin interneuron vulnerability could help to identify new targets for drug development. The ability to prevent or restore parvalbumin interneuron-related changes in injury and disease could alleviate some of the symptoms associated with the injury or disease. This is especially true for working memory, where it is within the realm of possibility that administration of a GluN2A-selective NMDA agonist could help to restore normal gamma oscillations and improve working memory in patients with schizophrenia.

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Molecular mechanisms and outcomes of a complex signaling system

Nicholas Smith

Abstract

Meal ingestion provokes the release of hormones and transmitters, which in turn regulate energy homeostasis and feeding behavior. One such hormone, glucagon-like peptide-1 (GLP-1), has received significant attention in the treatment of obesity and diabetes due to its potent incretin effect. In addition to the peripheral actions of GLP-1, this hormone is able to alter behavior through the modulation of multiple neural circuits. Recent work that focused on elucidating the mechanisms and outcomes of GLP-1 neuromodulation led to the discovery of an impressive array of GLP-1 actions. Here, we summarize the many levels at which the GLP-1 signal adapts to different systems, with the goal being to provide a background against which to guide future research.

Keywords: Bariatric surgery; Feeding behavior; Glucagon-like peptide-1; Reward

The neuroscience of evidentiary rules: The case of the present sense impression

Christopher Sundby

Abstract

This review aims to illustrate how neuroscientific measurements of brain activity can help formulate evidentiary rules that better reflect the realities of human cognition. The current rules are premised on untested psychological assumptions. This review examines the scientific validity of the assumptions underlying a provision of the Federal Rules of Evidence (FRE): the Present Sense Impression (PSI), codified as FRE 803(1) and adopted in 80% of states. The PSI is one of the exceptions to the ban against hearsay that has been adopted because it is believed that in specific circumstances, the risks of deceit and errors in memory and perception associated with hearsay are lessened. This belief, however, is premised on the “folk psychological” assumptions that: (1) contemporaneity does not give time for an individual to lie; (2) contemporaneity prevents memory errors; and (3) third-party listeners can detect contemporaneous lies. This review examines each of these assumptions and concludes that while there is solid evidence behind the second assumption, the current state of the science cannot directly answer the validity of the first and third assumptions. The review ends with a call for research targeted at assessing the validity of these assumptions given the potential implications for both this rule and for the broader role of neuroscience as an aid to legislators in assessing additional evidentiary and procedural rules.

Keywords: event related potential, electroencephalograph, law, lie detection, visual working memory, error positivity, error related negativity

Introduction

Since their adoption in 1975, the Federal Rules of Evidence (FRE) have governed all evidentiary decisions in federal courts, controlled the admissibility of evidence at trial, and determined what is permissible for the jury to consider in making its factual determinations1. The FRE constitute one of the legal system’s most important and complex sets of procedural rules and are designed to ensure the fair and impartial administration of justice by balancing accuracy, efficiency, and legitimacy2-4. If the rules fail to strike the proper balance, undesirable effects may follow: (1) the jury may never see evidence critical to accurately determining a case outcome5; (2) they may hear unduly prejudicial evidence that reduces the chance of an accurate outcome6; (3) trials may become longer and more burdensome and slow the system to a crawl7, or (4) the public may lose confidence in the system if they believe that the jury is basing their decisions on improper considerations8. The effective, efficient, and fair administration of evidentiary rules forms the foundation of the adversarial trial system9.

Despite the FRE’s role as a cornerstone of the trial system, many of the rules are based upon untested assumptions about how people process information based on “folk psychology”8,8. Examples include FRE 803 (2)’s excited utterance exception8, FRE 803(4)’s statements made for medical treatment exception10, FRE 804 (b)(2)’s dying utterance exception11, FRE 609’s limits on use of past criminal conviction evidence12, and FRE 403’s “undue prejudice” standard13.
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This review examines one of the exceptions to the general ban against hearsay (codified in FRE 80119), FRE 803(1)5, or the Present Sense Imposition (PSI). Hearsay evidence (i.e., out-of-court statements introduced into evidence to prove the truth of the matter asserted9) is generally banned because of the lack of traditional safeguards (e.g., witness being under oath, in the personal presence of the trier of fact, and subject to cross-examination6) against the dangers of testimonial evidence (e.g., errors in perception, memory, and narration and deceit11,17). The PSI is an exception to this ban when: (a) the statement describes the event in question, (b) the declarant perceived the event in person, and (c) the event and the statement were “substantially contemporaneous8.” This exception is generally thought to be premised on the three assumptions that contemporaneity limits a person’s ability to lie, reduces the risk of memory errors, and that third-party observers are better able to detect contemporaneous lies than lies about past events19-20 (see Figure 1 for a summary of the traditional safeguards and the assumed safeguards of the PSI against the dangers of testimonial evidence). This review will discuss the current state of the science underlying each of these assumptions in turn.

Figure 1: A comparison of the traditional safeguards and the PSI’s assumed safeguards against the sources of error in testimonial evidence. Any of the four sources of error in testimonial evidence (i.e., memory error, perception error, narration error, deceit) can lead to the admission of false testimony. The green text displays the traditional safeguards against these errors, including the witness being under oath, the fact finder (jury or judge) as a lie detector, and cross-examination. The red text denotes the PSI’s assumed safeguards, including no reliance on memory, the presence of a third-party listener as a lie detector, and insufficient time to create a lie.

The literature behind the PSI assumptions

*Ability to lie.* A lot of research has been done on the process of lying and on lie detection, but little research has directly assessed the effects of temporal delay between the perceived event and recitation of the lie21-22. The general process of lying is thought to involve inhibiting the truthful response, generating an alternative version, and holding two versions in working memory while regulating emotion21,23. Several fMRI imaging studies have associated these cognitive steps of deception with the medial frontal cortex (MFC), dorsolateral prefrontal cortex (DLPFC), ventrolateral prefrontal cortex (VLPFC), and anterior cingulate cortex (ACC)24-25. A series of EEG studies has begun to pull apart the time course of this potential deception circuitry and has associated several ERPs with some of the steps involved in generating a lie (summarized in Figure 2). Studies have found differences in early ERP components, including a reduced pro-responsive potential (PRP) 100ms prior to response during deception trials (associated with strategic monitoring and conflict resolution)26 and increased amplitudes in the early attention and executive function related ERPs N1 and N2 (also thought to reflect response monitoring)27. Differences have also been observed in late, post response, response monitoring, and conflict detection ERPs with increased negativity in the medial frontal negativity (MFN) 0-100 ms.
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post-response and reduced amplitude in the parietal late positive component (LPC) as late as 500-700ms post-response. These experiments have begun to tease apart the time course of lie generation and have identified useful markers of brain activity for future studies.

The N1 is believed to index the orienting of attention to task-relevant stimuli. The increased N1 found in deception trials may indicate greater attentional demands when generating a deceitful rather than truthful response about a stimulus. The stimulus-locked N2 is larger on incongruent than congruent trials in conflict tasks. Some researchers have suggested that the N2 and the error related negativity, discussed below, are related and index the same cognitive processes of error and conflict monitoring.

The MFN and the closely related error-related negativity (ERN) are both believed to reflect cognitive-control functions and have neural generators within the ACC. While there is some evidence for distinction in the underlying cognitive processes, other researchers have suggested that they are synonymous. At a minimum, both are linked to response monitoring and error detection, with both increasing with the degree of response conflict created by the stimulus. Deception studies in which the participant was shown the target stimuli before being cued to lie have found a reduced increase in the MFN between deception and truth telling, suggesting that when participants can evaluate the stimuli before preparing their response they can reduce the subsequent conflict between the stimuli and their deceitful response. Other studies have found increased amplitude in the MFN during deceit regardless of whether the conflict between the stimuli and intended response is perceptual or conceptual or whether the stimuli had been previously practiced or not. These findings suggest that contemporaneous lies may induce a greater increase in MFN or ERN amplitude than when the lie and the event are temporally divorced.

Most researchers view the LPC as merely different nomenclature for the canonical P300 ERP. The majority of studies examining the P300 in deception have utilized the Guilty Knowledge Test, which consistently finds an increased amplitude in the P300 in response to previously seen, but told to conceal, stimuli. As such, the P300 is one of the more promising ERPs for the development of lie detection tests, but seems unlikely to vary with changes in contemporaneity and does little to illuminate the cognitive processes behind lie generation.

While no studies have assessed the effects of temporal delay between viewing the event and relating the lie, several studies have examined the time it takes to generate a lie. While substantial variation exists, most studies converge around an additional 200–400 ms for lying compared to truth telling. This small of a delay in reaction time raises obvious concerns over the validity of the PSI’s underlying assumption that contemporaneity does not give sufficient time for an individual to lie. If individuals can fabricate lies in less than half of a second, perhaps contemporaneity is not as strong of a safeguard as the rule and many judges assume. Importantly, however, these studies often test previously instructed lies, simple yes or no lies, or lies told with significant advanced notice. It may be that lying within a coherent story line, where the fabricated

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**Figure 2: A timeline of ERPs implicated in deception.** This diagram depicts temporal relationship of stimulus- and response-locked ERPs that have been implicated in lie generation. Four stimulus-locked ERPs have been found to differ significantly in amplitude between deceit and truthful conditions. The amplitude of the N1 and N2 have been found to significantly increased in deceitful compared to truthful conditions, and the P3 and parietal late positive component (LPC) have been found to be significantly decreased. Two response-locked ERPs have been found to significantly vary between deceitful and truthful conditions with both the pre-responsive potential (PRP) and medial frontal negativity (MFN) being significantly reduced in the deceitful compared to truthful condition. Note that these results come from varying experimental paradigms.
element must be assimilated into the truthful elements, takes significantly longer than simply reciting a pre-instructed lie or lying with advance notice, potentially leaving intact the rule’s underlying assumption about the difficulty of deceit under true contemporaneity.

Several studies have recognized the important differences between lie types and have begun to examine the differences between them. For example, a study by Ganis et al. hypothesized that isolated spontaneous lies and memorized lies recited in a coherent scenario would employ different cognitive mechanisms, with spontaneous isolated lies drawing on semantic and episodic knowledge, and memorized lies recited in a coherent scenario relying only on episodic knowledge but requiring a higher working memory load since the subjects would have to maintain and crosscheck more details. Episodic knowledge refers to retrieval of a past specific episode for reference, while semantic knowledge refers to the generation of a plausible answer set from general information without reference to a particular time or place. The study found several areas with increased activation in both deception types, suggesting at least some common circuitry to deception, as well as several differences, suggesting that there may be some differences in how individuals spontaneously generate isolated lies and how they recite memorized lies within a coherent scenario. They did not, however, find any behavioral differences between the conditions, with no significant differences in error rate and they did not measure reaction time.

Other researchers have examined alternative categorizations of lies. A Hu et al. study, for example, compared brain activity between self-referential and other-referential lies using EEG. The researchers found a similar pattern to Ganis et al., with certain ERPs common to both types of deception, but others distinct to a specific lie type, further suggesting some partial differentiation in the underlying cognitive processes. One of these observed differences, an interaction between stimulus and response types on N2 and P3 ERPs, suggests that some types of lies, here self-referential lies, may be more cognitively demanding than other lie types.

Memory errors. True contemporaneity as a safeguard against memory errors is well supported in the literature. True contemporaneity reduces the risk of memory errors because witnesses draw from working memory without the need for long-term storage or retrieval. Visual working memory allows for the temporary storage of visual representations so that these representations can be manipulated, encoded into a more durable type of memory, and used to guide behavior. Not all information attended to and maintained in working memory is encoded in long term memory (LTM). Whether an object is encoded into LTM depends on the degree of manipulation or engagement with the representation. Once encoded in LTM, a memory must be retrieved and maintained in working memory to recall and relate an episodic memory. This step of retrieval and recall adds an additional source of potential memory error not present under true contemporaneity and these errors can be preserved in the representation recommitted to LTM.

While the risk of retrieval errors is largely eliminated with true contemporaneity, other sources of memory error still exist. A witness’s working memory capacity, for example, could be exceeded. Working memory capacity has been consistently measured at 3-4 objects, regardless of object complexity or the number of object features. Memory errors could also manifest if the witness did not focus their attention on the important aspect of the event. For example, numerous studies have documented the “weapon focus effect” where witnesses are focused on the weapon rather than the features of the perpetrator of a crime and are significantly less accurate at identifying perpetrators in weapon-present versus weapon-absent crimes. Some researchers have suggested that the capacity limits of working memory and attentional limits are one and the same, with working memory representations being maintained by actively focusing and rapidly switching attention between the representations.
A memory error could also occur if a court’s definition of “substantially contemporaneous” extends beyond working memory’s temporal limits6-17. A survey of published cases reveals evidence being admitted under the PSI with a temporal range between a few seconds and up to twenty-three minutes19,62-63. The court’s focus on the temporal delay reflects an antiquated conceptualizing of attention and memory. A more appropriate focus would be whether the witness has actively maintained a representation of the event in working memory from the moment of observation until the moment of relating the event64-66. Working memory representations appear to be maintained by the synchronized firing of neurons in a specified pattern, and possibly using synchronized oscillations as a carrier signal, though the exact nature and interaction between these signals still needs to be fully explored17. Temporal delay is an imperfect proxy for this maintenance.

*Ability to detect lies.* The legal system relies heavily on the jury as a lie detector and the PSI assumes the ability of a third-party listener to detect lies67,19. However, a meta-analysis of 206 studies assessing the ability of untrained laymen to detect lies found only a 47% detection rate of lies and a lie-truth discrimination of 54%, or just over that of a coin flip68. The authors do note, however, that this actually represents a nontrivial effect size producing a mean difference of approximately 0.40 standard deviations in judgments of lies versus truths. The study also found that participants were equally able to detect lies conveyed via audio only and audiovisual. Additionally, lies told by senders who were highly motivated to deceive were detected at a significantly higher rate than when motivation was low, but only when conveyed via a video or audiovisual medium, with no effect on audio only transmission59.

The meta-analysis found no effect of expertise on lie detection rates, defined as any profession whom the original researchers deemed expert, for example judges or law enforcement personnel9. Other studies, however, have found increased accuracy for some experts, with one study finding a 64% accuracy rate for Central Intelligence Agency Officers69. Other studies have focused on training aimed at specific “tells,” rather than expertise inherent or impugned to specific professions. For example, there has been a long-standing interest in “micro-expressions” as “tells” of deceit70. A further distinction has been made between “subtle expressions” (defined as fragments of typically suppressed or masked affect displays using only a subset of the normally and reliably associated musculature with minimal voluntary control71) and “micro-expressions” (defined as full muscular expressions of affect that occur for only a brief instant, typically around 1/25th of a second, such that most observers will fail to consciously perceive them72). However, one study that compared lie detection performance before and after training on both the Micro Expression Training Tool (METT) and Subtle Expression Training Tool (SETT) found no correlation between performance on either the METT and SETT and detection of unemotional lies, but a significant positive correlation between performance on the SETT and reported reliance on facial expressions and detection of emotional lies73. Another study examined the role of auditory “tells” of deception by filtering out certain cues74. The study found some evidence for increased detection of lies through changes in intonation, voice quality, and rhythm, though the level of manipulation to the audio (e.g., playing it backwards) makes it difficult to interpret65.

The overwhelming majority of these studies, however, have found that participants perform no better than chance at detecting deceit61. Most pertinent to the PSI’s assumption, the meta-analysis did examine studies that varied the level of preparation time to lie and found mixed results, with the within-study evidence suggesting that prepared lies were harder to detect, but the between study evidence suggesting that preparation led to higher detection59.
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Conclusion

A survey of the literature makes clear that further research is needed to directly assess the validity of the assumptions underlying the PSI. The reviewed literature, while informative and allowing for the formation of hypotheses, does not establish whether contemporaneity is or is not a safeguard against deceit, provides little basis for deducing when contemporaneity ends, and does not provide a definitive answer to whether third parties can reliably detect contemporaneous lies. Future studies need to probe three questions while also improving ecological validity: (1) whether contemporaneity is a sufficient safeguard against deceit; (2) if so, at what time point does this safeguard cease to be effective; and (3) are individuals more capable of detecting lies about contemporaneous events than past events.

Directly testing these assumptions could have a profound impact on the PSI rule. If the assumptions are proved valid, the rule may enjoy increased legitimacy. Furthermore, a better understanding of the nature of the contemporaneity safeguard may result in a more consistent application of the “contemporaneous” requirement. If the assumptions are proved false, there are several possible scenarios. Policy makers may choose to keep the rule due to a lack of viable alternatives, they may consider a change but opt against it out of an efficiency interest or alternative motive, or they may craft a new rule based on a more sound understanding of the cognitive processes underlying the production and processing of lies about contemporaneous events. More broadly, if neuroscience can enrich the conversation around the PSI, it may be able to do so for many additional evidentiary and procedural rules. Should this prove to be the case, neuroscience should be looked to as a valuable tool in assessing and improving procedural rules premised on cognitive processes.

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Common and unique auditory, visual, and audiovisual neural representations

David Tovar

Abstract
In the past decade, neural decoding has opened new avenues to investigate the way in which the brain encodes information. These advances have made it possible to dream about “mind reading” and the possibility of “cracking” the “neural code”. However, the “neural code” initially studied was driven decidedly by investigations of the visual system, with relatively fewer studies of the other sensory systems. Questions regarding how sensory systems encode information separately, and how they may complement each other were largely unexplored until recently. Answering these questions will provide a richer understanding of how the brain acquires and utilizes sensory information in both physiological states as well as during pathological insults. Furthermore, studying the visual system within the context of other sensory modalities will help further our understanding of visual representational geometry at both the individual as well as group level.

Introduction
As graduation season approaches, happy graduates from across the country are enjoying visits from parents and receiving phone calls from distant relatives. Throughout these experiences, these proud graduates will seamlessly recognize the voice of their grandmother on the phone and the face of their mother proudly looking on during graduation. Their ability to recognize their family members despite the family members’ respective identity being transmitted through different sensory modalities reveals a foregone truth; we fundamentally live in a multisensory world and encode the people and objects in our environment through a number of different modalities. In the past decade, neural decoding techniques have increased our understanding of how visual and auditory information are processed in the brain and have begun to reveal the means by which these sensory modalities interact in order to produce perceptual experience. Perceptual experiences have been framed under the concept of brain states or neural representations. The content of the neural representation can include a sight, sound, smell, taste, emotional state, or a goal\(^1\). The relationship between these neural representations can then be conceptualized in a representational map or geometry. In this review, I will explore key challenges in object recognition in the visual and auditory system, how studies have used neural decoding to provide insight into these challenges, the interaction between the auditory and visual system, and identify opportunities for further research.

Visual challenges
Stable object identity in vision requires an object to be invariant across a number of different conditions. For example, in recognizing a friend, one needs to have size invariance or the ability to recognize a friend whether she is far away or close. One would also need position invariance (i.e., the ability to recognize a friend whether she is in the right or left visual field) as well as rotational invariance (i.e., the ability to recognize a friend despite the fact that she has turned her head slightly). It would not be beneficial if every time a friend moved, the ability to recognize her was lost. Solving the problem of invariance is difficult because for any given object, a 300ms glance involves 100 million retinal photoreceptors and 1 million ganglion cells, leading to
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1 million ganglion cell representations\(^2\)-\(^4\). Encountering a friend can activate several of these representations depending on position, size, luminance, and viewpoint, amongst other properties. The face of an enemy can also activate one of these 1 million representations. Often times, the ganglion representations that are activated will overlap. If photoreceptor and ganglion cell activation for two completely different people are at times indistinguishable, how then does the brain solve this problem?

**Higher order visual representations**

Invariant visual representations in the brain were initially thought of in a modular manner wherein areas of the brain activate specifically for object categories. In this manner, the parahippocampal place area (PPA) got its name because of its association to code for places and scenes and the fusiform face area (FFA) for its association with faces\(^5\)-\(^6\). However, since their discoveries, the tight association between these areas and the objects they were named after has come into question. Evidence has emerged that the PPA may function to establish contextual relationships between objects and the FFA functions to establish expertise\(^7\)-\(^8\). The conflicting views have led to debates on the specialization of these modules that are still active to this day\(^9\)-\(^10\). While the type of modularity present within the brain is still in question, it has become increasingly apparent through further research that the brain utilizes a distributed code along with discrete brain areas to establish object identity\(^11\)-\(^15\).

**Representational similarity analysis in vision**

The similarity or dissimilarity of the distributed codes has been subsequently used to build visual representational spaces (Figure 1). In the studies to first conceptualize representational spaces, subjects were presented with a wide array of objects with categories spanning faces, bodies, natural objects, and manmade objects\(^19\)-\(^20\). The stimuli were structured hierarchically with individual exemplars, subordinate categories such as human faces and animal faces, intermediate categories such as faces and bodies, and superordinate categories such as animate and inanimate object. The neural responses as subjects viewed these images were then grouped using multidimensional scaling. In this framework, objects with more similar neural responses are grouped together in representational space while objects with more dissimilar neural responses are grouped further apart in space. Structurally, the space largely adopted the categorical structure of stimuli with subordinate categories grouping together and superordinate categories joining the subcategories together\(^19\)-\(^20\). Remarkably, the grouping in representational space between humans and macaques was similar despite different ecological needs\(^20\). These similarities demonstrate a possible common code by which information is stored in the brain.

In addition to spatial structure, the representational space has also been shown to have a temporal structure, organizing category information as a function of time. Utilizing magnetoencephalography (MEG), studies found that different levels of category abstraction have different optimal readout times with subordinate
objects generally arriving in the brain before superordinate objects (approximately 40-80ms faster)\textsuperscript{21,22}. Using discriminant cross training\textsuperscript{23}, a technique in which the classifier is trained during one time period and tested in subsequent time periods, Carlson et al.\textsuperscript{21} were also able to show evidence that object information degrades differently in different parts of the brain. The working assumption in their study was that visual information arrives at different times within the brain, and as such, training the classifier at earlier time points is indicative of early visual areas and classifier training at later time points (approximately 110ms for inferior temporal (IT) cortex\textsuperscript{2}) is indicative of later visual areas. As expected, the classifier trained in the time periods associated with information arrival at IT cortex was more generalizable than the classifier trained on earlier time periods. The results from this study were validated by an interesting study by Cichy el.\textsuperscript{22} which used MEG and functional MRI (fMRI) together to specifically relate the dynamics of representations with spatial location. They performed representational similarity analysis (RSA) in fMRI regions of interest in both primary visual cortex (V1) and IT cortex and compared the dissimilarity matrices with the dissimilarity matrices acquired from MEG signal. They found that the RSA of IT cortex correlated with a larger range of the MEG dissimilarity matrices when compared to the dissimilarity matrices of V1. Together, these findings show that the brain organizes information in both a spatial and temporal population code.

Interestingly, both spatial and temporal studies into visual representational geometry have demonstrated a distinct categorical distinction for animate and inanimate objects in IT cortex as well as the amygdala\textsuperscript{19-22,24}. The animate/inanimate category boundary has been used to show that representational spaces are perceptually relevant, linking spatial and temporal properties of representational space with behavior\textsuperscript{25,26}. Using the relative distances of objects from the animate inanimate category boundary, studies have been able to make predictions on categorization reaction times\textsuperscript{25,26}. Namely, objects closer to the animate inanimate category boundary are more difficult to distinguish as animate or inanimate and, as such, will have longer reaction times. Conversely, objects far apart from the category boundary are easier to distinguish as either animate or inanimate and, as such, have shorter reaction times. These predications fall within the framework of perceptual decision-models which state that evidence close to a decision boundary is more ambiguous, resulting in more decision time, while evidence far from a boundary is less ambiguous, resulting in more rapid decisions\textsuperscript{27-29}.

Curiously, these studies only demonstrate a relationship between animate distance to category bound, but do not demonstrate a relationship between inanimate objects and their distance to the category bound. This result may reflect an ecological importance to be able to distinguish animate objects in the environment\textsuperscript{26}. However, an alternative explanation is that animate objects when compared to inanimate objects do not involve other sensory modalities. For example, seeing a man may at some level activate the representation of a man’s voice. Inanimate objects on the other hand are often times restricted to the visual modality. It is difficult to describe what celery “sounds” like. Therefore, more work is needed to understand the perceptual relevance of animate objects. Specifically, is their relevance driven by a top-down driven process (i.e., living things are important to distinguish) or is it a bottom up driven process (i.e., living things have a richer sensory profile than non-living things)? Additionally, the role that other sensory systems have in modifying vision have gone largely unexplored\textsuperscript{1}. These avenues of research are especially important in the context of recent studies\textsuperscript{30-32} which have shown that realistic stimuli, such as full action movies (e.g., Raiders of the Lost Ark), provide a sufficiently strong stimulus set to create a between subject classifier. These studies are able to use neural data from a cohort of subjects to “train” a classifier that can then decode information from a “test” subject’s brain.
Auditory challenges

In audition, an object can be thought as the computational result of the auditory system’s capacity to detect, extract, segregate, and group spectrotemporal regularities in the acoustic environment\(^3\). However, the major difference between audition and vision is that, unlike the visual scene, resolving auditory objects crucially depends on the temporal properties of the stimuli. In this sense, auditory invariance introduces the physical property of time with temporal integration becoming a larger component of perceptual grouping. The temporal aspects of the sounds become part of the auditory systems’ “stimulus manifolds” for different sounds. Despite these differences, auditory objects share many properties to the visual manifolds. We untangle a violin from a bassoon despite overlapping properties. A violin will sound like a violin regardless if a single high note or a rapid melody is played, whether it is played loudly or softly, or whether it is played alone or as part of an orchestra\(^3\). Therefore, the percepts must at some level be disassociated from its physical properties\(^3\).

Higher order auditory representations

Much like in early vision science, the auditory system has been primarily defined through a modular approach. It has not been until the past decade that studies have begun finding where in the auditory system invariant auditory objects are encoded. Complete auditory objects have been found to be encoded in anteroventral temporal cortex, including the superior temporal regions which encode for familiar voices similar to the FFA’s affinity for faces in the visual system\(^9\). Other areas in the anterior superior temporal regions are more specific to auditory objects with clusters selective to musical instrument sounds\(^\)\(^3\). On the other hand, the posterior superior temporal regions are more selective to lower level auditory features\(^\)\(^7\). Yet other studies have found regions for object categories including complex natural speech comprehension, singing, and piano playing in anterior parts of the supratemporal regions with areas located in the posterior regions having a greater involvement in audition related to action\(^3\). In addition, studies have shown that audition has simple and complex cells and dorsal “where” and ventral “what” streams for information processing. These systems are part hierarchical processing starting with a tonotopic map and ending in object selective regions\(^9\),\(^\)\(^9\). Like the visual system, these object areas can be independent of physical properties, as shown in hysteresis studies. These studies have shown that “bad” and “dad” sounds morphs activate selective primate neurons in the belt region of auditory cortex according to percepts with identical morphs as reflected by behavioral measure.

Studies utilizing MVPA have been able to show that, like the visual system, auditory objects are coded with both modular and distributed representations\(^1\),\(^2\). Auditory sounds belonging to different humans in an experiment can be decoded without the use of A1. The identity can be decoded in amodal areas of the brain, such as the superior temporal sulcus. Distinct activation patterns are seen for both the voice and the content of the voices\(^1\). These findings suggest that an abstract representation of content and speaker of that content is present in auditory regions as well as amodal regions\(^2\).

Representational similarity analysis in audition

While the representational geometry of visual objects has been well studied, studies investigating the representational spaces for audition are comparably scarce\(^1\). The reasons for this disparity may be several. The visual cortex utilizes about 50% of cortical resources\(^4\). As such, there may just be more visual system to study. Along the same vein, visual information dominates attentional resources when presented in unison with either auditory and haptic information\(^4\),\(^6\). The more practical possibility however is that RSA was first developed with visual studies, and the information that can be gained with RSA in other sensory modalities is waiting to be discovered.
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One of the challenges that RSA has been helpful in include parsing out higher level and lower level auditory properties\textsuperscript{35}. Giordano et al.\textsuperscript{47} utilized MVPA to create a dissimilarity matrix for a wide array of sound objects and a separate dissimilarity matrix for different physical properties of sound (e.g., timbre, loudness). These values were then subtracted from the object driven fMRI activity, and searchlight analysis was used in order to find the areas within the brain that were specific to abstract stimuli categories and not stimulus properties. These areas included the right planum temporale and posterior temporal gyrus, matching some of the category selective regions denoted in univariate modular approaches\textsuperscript{38,48}. An RSA study by Teng et al.\textsuperscript{49} found that sound source (e.g., horn, bell) and the acoustic space in which the sounds are occupy unique patterns within the brain\textsuperscript{49}. This study showed that different attributes of sound are encoded in parallel within the brain. These studies, along with others that show diverse sound categories along the superior temporal sulcus\textsuperscript{50}, hold promise for further characterization of auditory representational geometry.

Interaction between audition and vision

The superior colliculus was among the first multisensory areas discovered in the brain\textsuperscript{51}. Its function is to primarily code for directionality. By using more than one sensory modality, the superior colliculus benefits from superadditive effects, producing a signal that is greater than individual modalities added together and furthermore demonstrates increases in the reliability of the signal from trial to trial\textsuperscript{52}. A growing amount of literature suggests that the cortex also has areas of multisensory integration that code for higher order representations\textsuperscript{53-55}.

Much like vision research and auditory research, multisensory studies have begun by searching for shared brain areas across sensory modalities that code for object representations. Initial studies utilized the well-known fMRI adaptation effects where repetitive presentation of an object will lead to attenuation of the blood oxygen-level dependent (BOLD) signal\textsuperscript{56}. If there are areas in the brain that receive higher order sensory information across sensory modalities, then repetitive presentation of objects across sensory modalities would similarly show an attenuation effect. Studies have found this effect for faces in superior temporal sulcus\textsuperscript{57}. Multivariate studies have also been useful in for other areas of convergence. A study by Man et al.\textsuperscript{58} used a series of three objects which subjects saw, felt, and heard while in an fMRI scanner. They then performed crossmodal decoding, where they would train a classifier on the subject’s fMRI activity while they were presented with one modality and then tested on the fMRI activity when the subject experienced another modality. From this study, it was found that the right tempo-occipital junction was invariant to audiovisual stimuli, the left postcentral gyrus and parietal operculum were invariant to audiotactile invariance, and the right postcentral and supramarginal gyri were invariant to visuotactile stimuli. Aside from showing that the modalities share common neural substrates, in a somewhat of an incidental finding, they found that their multisensory functional localizer revealed a multimodal potentiating effect when compared to the respective unsensory localizers. This finding shows that the cortex is likely superadditive when combining sensory information from more than one modally.

The ability to crossmodally decode object identity agrees with a host of multisensory research that shows a shared common distributed code between sensory modalities. Studies have found that auditory information can be found in visual and somatosensory cortices\textsuperscript{59-61}. Similarly, visual information can be decoded in auditory and somatosensory cortex\textsuperscript{62,63}. However, what is the significance of such findings? Many point to these amodal representations as possible neural bases for mental concepts. Unfortunately, many of these tasks focus on simple recognition of the object without a way of linking integration effects in the sensory cortex with behavior. Furthermore, many of these tasks also introduce the possible confound of introducing top-down effects while they are presented with stimuli. Subjects are often told to “imagine seeing in your mind’s eye the images that go with these sound clips as vividly as possible” or “imagine hearing in your mind’s eye the
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sounds that go with these video clips as vividly as possible."\textsuperscript{62,64} Previous studies have shown that subjects who report more vivid imagery also have better crossmodal classification\textsuperscript{65}. This type of confound may not be insignificant considering the host of different studies which have looked at the role of mental imagery and expectations (through the use of a cue) on activating category selective regions\textsuperscript{66–70}. Interestingly, recent studies have found that the modality in which a subject is asked to imagine primarily activates that sensory modality’s specific machinery (i.e., activating FFA if told to imagine a face)\textsuperscript{71}. Yet other studies have found crossmodal activation within top-down activity, with primary visual cortex secondarily activated from auditory imagery\textsuperscript{59}.

Invariant representation for different types of categories have also begun to be localized to different regions in the brain. Audiovisual representations for emotional categories have been found in the left posterior superior temporal sulcus (pSTS) and medial prefrontal cortex. Meanwhile, the right pSTS has been found to be an invariant region for crossmodal person signals\textsuperscript{72,73}. These crossmodal person signals have been further characterized in a multivariate analysis looking at within face and within voice analysis. Activation in the area is able to discriminate between faces and between voice, forming a general identity map\textsuperscript{74}. Studies using retrograde tracers in monkeys have shown that the pSTS is gathering information from the anterior temporal lobes, a network that has voice and face representation of person identity\textsuperscript{81,75}. Concordantly, several studies have found functional and anatomical changes in the STS in autism\textsuperscript{76}, providing potential insight into the pathophysiology of autism.

Multisensory open questions

Much of multisensory research into higher order representation has broadly asked if there are areas in the brain responsible for integrating information across sensory modalities. Much progress has been made in finding different areas of the brain that may hold different types of amodal representations. However, broader studies to characterize the representational geometry of multisensory objects have not been conducted, as they have been in vision and auditory research\textsuperscript{19,20,47}. The lack of a multisensory representational space may be due to reduced number of stimuli used in studies, with some studies having as few as 3 multisensory stimuli\textsuperscript{58}. A lack of a representational space for multisensory objects prevents us from being able to ask a number of questions about how the representational space can be modified, as listed below.

General questions regarding multisensory potentiation effects for object recognition:

1. Does having information from several sensory modalities improve behavioral performance and reliability?
2. Are these potential behavioral benefits reflected through expansion of representational space?
3. How much does each respective sensory modality contribute to expanding the representational space?
4. Is this expansion the same for each subject?
   a. Are auditory and visual contributions to a multisensory percept similar between subjects?

Question regarding respective stimulus quality in different modalities and multisensory effects:

1. Do the superadditive effects on the representational geometry increase with decreased stimulus quality?
2. Do object closer to categorical boundaries in visual studies\textsuperscript{20,26} benefit the most from added auditory information?
3. Do top-down processes (attention or expectations) affect the unsensory representational space\textsuperscript{27} and multisensory representations in the same manner?
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Additional questions regarding the temporal dynamic of multisensory object recognition:

1. Does visual information provide the initial wave of activity for object and does this activity in turn activate auditory higher order representations?
2. Alternatively, does auditory and visual information produce a higher order representation that arrives at approximately the same time?
3. Does multisensory integration at the object level produce a temporal shift in the feed forward sweep of information?
4. Do primary sensory cortices become sensitized by other sensory modalities with added object sensory information?

Figure 2 shows a possible result from an audiovisual representational space that would answer some of the questions posed.

Clinical significance

Finding areas in the brain that are responsive to multisensory stimuli and defining the multisensory representational space and its dynamics may be esoterically interesting. But what is the significance of such findings? These findings become meaningful because, beyond just providing us with a greater understanding of how the brain functions, they begin to tell us more about disease processes. These disease processes can disrupt the ability to build and integrate across stable sensory representations. What might these diseases be? Disease may be defined as “the human subject striving to preserve its identity in adverse circumstances...a chaos induced in the first instance by destruction of important integrations, and reorganized on an unstable basis in the process of rehabilitation” (Ivy McKenzie). It is both the initial insult as well as the compensatory effects of the body to mitigate the damage that leads to pathological states. Negative compensatory mechanisms are a common occurrence in medicine. An infection can quickly lead to sepsis and then death, not necessarily because of the virulence of the bacteria, but because of the massive cytokine release from the body’s immune system. The treatment in these cases is to treat the offending bacteria with antibiotics, but critically also treat the maladaptive physiological vasodilation response which is depriving the body’s organs of oxygen. Why then, should the brain be any different?

The difficulty with neurological and neuropsychiatric diseases is that unlike sepsis where the inciting offense is many times a known bacterium, it is often times difficult to identify the inciting factor and mechanisms. That said, there are cases where abrupt changes in behavior can be closely tied to an event, such as a clearly localized stroke. However, what makes neuropsychiatric disease difficult is that cognitive compensatory mechanisms are incredibly difficult to determine. Vasodilation in sepsis can easily be measured with a blood pressure cuff. It was only after physicians made the observation that many severely ill patients tended to have low blood pressure, that the cellular and molecular mechanism of cytokine and histamine release was uncovered. In neurological diseases, we are sorely lacking a “blood pressure cuff” that can inform us on the function of the brain and its response to insult. It is only after we understand how the brain encodes meaningful representations and responds adaptively or maladaptively after an insult that we will make significant progress into mechanisms of disease and treatment.
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There are a number of neurological and neuropsychiatric disorders that may especially benefit from a greater understanding of multisensory encoding of object. In the newest DSM V\textsuperscript{82}, one of the new features that has been added to autism is “hypo- and hypersensitivity and unusual interest in sensory aspects of the environment”. This feature is not unique to autism, as adults with ADHD also express hypo- and hypersensitivity\textsuperscript{83}. One of the areas that has most been implicated in autism is the superior temporal sulcus, an area associated with multisensory integration and modality invariant person identity and animate objects. Interestingly, patients with autism also have impaired identification of animate objects with oftentimes improved identification of inanimate objects and increased reliance on vision when compared to other modalities\textsuperscript{84–86}. These sensory abnormalities may be responsible for many of the behavioral symptoms found in autism. In order to understand the sensory deficits, it will be necessary to find out whether deficits in multisensory integration are due to deficits in other sensory modalities or whether the multisensory deficits lead to deficits in sensory modalities.

On the other end of the spectrum, there are several circumstances of sensory systems adaptively changing to losses in other sensory systems. Considering the large amount of interaction between sensory modalities, perhaps this should not be surprising\textsuperscript{87}. In the absence of visual information, as seen in blind individuals, the occipital lobe processes auditory, haptic, and olfactory stimuli\textsuperscript{88–90}. Patients with acquired prosopagnosia develop superior voice recognition after their lesions\textsuperscript{91}. It is unknown whether these changes are due to enhancement of auditory specific voice recognition areas or if they are due to changes in the way sensory information is integrated\textsuperscript{92}. A multisensory study using representational space analysis may be able help answer this question and also determine the plasticity of representations across sensory systems.

Conclusion

In accomplishing object recognition that is fast, accurate, and reliable, sensory systems must adopt functional and structural strategies such as parallel processing, incorporation of other sensory modalities, and distributed coding, which make the challenge of binding all of this information paramount. Several different areas of the brain have been identified as object selective areas, invariant to lower level sensory modifications. However, the current multisensory studies have not utilized many of the tools that have been developed for unisensory experiments. By developing a multisensory representational space, our understanding of how the brain’s sensory systems respond adaptively or maladaptively after an insult will increase, guiding future research on mechanisms and treatment.

References
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