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LETTER FROM THE EDITORS

Dear Friends and Colleagues of the Vanderbilt Neuroscience Community,

It’s a pleasure to bring you another issue of Vanderbilt Reviews Neuroscience! This year’s issue contains 17 spectacular reviews from the 2014 qualifying class. As always, the topics are very diverse and include important subjects like endocannabinoid signaling in anxiety and addiction, multisensory processing, antioxidants in Alzheimer’s disease, the role of the extended amygdala in diet failure and alterations in sensory processing and neurochemistry in Autism Spectrum Disorder. I felt especially privileged working on this issue after serving as an academic coordinator last year. Each of the candidates studied diligently and wrote very insightful reviews of their research topics. I’m very proud of their hard work and hope you are as well.

The 2014-2015 academic year was also a great time for the Vanderbilt Brain Institute. Neuroscience trainees published over 30 manuscripts, many of which appeared in prestigious journals. Several of these publications are featured in this issue’s Highlights and Briefs section. Outreach was very successful this past year, with events like Brain Blast, Music and the Mind and Brain Awareness month, which you can read more about in the Outreach and Education section. I’m also quite pleased to feature original artwork on this year’s VRN cover by one of our talented fellow graduate students, Joyonna Gamble-George.

This year’s editorial staff, consisting of Franklin Echevarria, Elaine Ritter and Kathryn Unruh, put forth a tremendous amount of effort in the creation of this issue. They spent hours writing, editing and communicating with the candidates to ensure that every review was in perfect condition. I am very thankful for all of their hard work! I am also greatly indebted to last year’s Editors-in-Chief, Barbara O’Brien and Tyne Miller, who helped me successfully navigate the editorial process from start to finish. Happy reading and congratulations to all of the candidates on the completion of their qualifying exams.

Your Editor-in-Chief,

Courtney Bricker-Anthony

From left: Kathryn E. Unruh, Courtney Bricker-Anthony, Franklin D. Echevarria, Elaine Ritter
Vanderbilt Brain Institute  
U1205 Medical Center North  
Nashville, TN 37232  
(615) 936-3736

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

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**2015 Editorial Board**

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Bruce Carter
A Message from the Director of the Vanderbilt Brain Institute

It is hard to believe that another year has passed and that another class of students has successfully transitioned into the realm of “doctoral candidacy.” What you read in these pages represents the culmination of a great deal of work by them in developing expertise within their chosen area of research, and once again I am very proud of the final product.

I’d like to characterize this past year as one of change, but change seems to be more the norm than the exception these days. Putting it in terms I understand, in the yin and yang of plasticity and stability, plasticity is winning! One of the most exciting opportunities that this year presents to us is becoming a more integral part of the broader university. As the VU-VUMC transition moves forward, we, like our basic science departmental colleagues, will become more tightly yoked to VU. For neuroscience, the transition will not only be a seamless one (since we already have such a strong transinstitutional footprint), but it will also be one that more readily allows us to explore and expand our interactions with other entities and disciplines on campus. With our thriving undergraduate neuroscience major, there is great opportunity in thinking about partnerships that create an integrated training landscape that spans the undergraduate, graduate and postdoctoral experiences. As many of you know, we already have formalized programs that bridge neuroscience with law and education, and discussions are ongoing to explore the synergies that undoubtedly exist with our colleagues in business, divinity, engineering and, music.

Although these days I often think of the dual-edged Chinese proverb “may one live in interesting times,” the opportunities in front of us provide me with tremendous enthusiasm for the future.

Yours in science,

Mark T. Wallace
A Message from the Neuroscience Program Director of Graduate Studies

Dear Readers,

It has been another exciting year of growth for the Neuroscience Program! We admitted 7 new students through the direct admit route and accepted 7 from the IGP and 2 MSTPs. These students come from a variety backgrounds and locations and represent a wide range of interests. In addition to our traditional neuroscience program, our new initiatives continue to thrive, with two of the students joining the Educational Neuroscience program and one is in the Law/Neuroscience program.

Our curriculum continues to evolve, with substantial input from the students. This year we revised our courses, including the addition of Neurobiology of Disease as well as revamping Neuroscience Discussions, to focus primarily on grant writing. We also developed a new Qualifying Exam process, which still includes writing a review that is published in the VRN.

I continue to be amazed at the accomplishments of our students, both scientifically and in their leadership roles. This year approximately 20 students successfully defended their PhDs, with many important and exciting discoveries that have significantly advanced the field of neuroscience. Our students also continue to organize our annual retreat, the Brain Blast outreach program, as well as other activities and events, including running this unique publication, which they founded. The reviews in the VRN are of the highest caliber and reflect the scholarly work and diverse interests of our outstanding students. They are always a pleasure to read and provide fascinating insights into the workings of the brain. It is a privilege to serve as the Director of Graduate Studies for such a remarkable group of students!

Sincerely,

Bruce Carter
An Update from the Neuroscience Student Organization President

As president of the Neuroscience Student Organization (NSO), I would like to congratulate the editors and contributors to Vanderbilt Reviews Neuroscience for another great publication.

The NSO has been in operation since 2000 and has continued to evolve in order to meet the needs of current neuroscience students. I have been honored to serve as the president of this outstanding group of young scientists. Although we are only halfway through the year, I would like to take this opportunity to highlight some of the amazing work that NSO officers have already done.

One of the most notable changes to occur has been the development of the new qualifying exam process. Led by our curriculum coordinators, Michael Tackenberg and Eric Wilkey, sweeping changes have been made to make the qualifying exam more consistent with other departments and less time consuming. Based on student input, improvements have also been made to the neuroscience curriculum to better support current students.

To ensure that current students survive the new qualifying process, the academic coordinators have done a fantastic job of helping them prepare. Shilpy Dixit, Raaj Gowrishankar, Alyssa Lokits, and Monika Murphy have put in a tremendous amount of work scheduling mock quals, meeting with students, and providing invaluable resources.

An important tenet of neuroscience education is learning how to reach out to the local community to facilitate an interest in science, especially the science of the brain. LeAnne Kurella and Chelsea Šnarrenberg have led the NSO in organizing Brain Blast, the student invited speaker, and spearheading new initiatives such as teaching at A Room at the Inn and the Lentz Public Health center. Look for lots of new volunteer opportunities in the upcoming year!

The retreat committee, Lauren Bryant and Daniel Kashima, organized the 2015 retreat at the Adventure Science Center, which will be an exciting new experience for all students, right down to the laser show! The retreat should be a great mix of science within and outside our fields. The invited speaker at this year’s retreat is Dr. Sheri-lynn Black, a neurobiologist from Duke University who specializes in identifying the most successful practices in STEM higher education— a cause that I think is close to all of us. I can’t wait to learn more from her.

Finally, as neuroscience students, we all know how important it is to balance our hard work with a bit of fun. Our social coordinators, Brandon Turner and Cassie Rettlaff have provided opportunities for current students to meet and complain about how tough our jobs are. I look forward to seeing all of you at future events, and don’t forget to join the NSO Facebook page to learn more about upcoming events!

I am so proud to have served as president for the past few months, and I can’t wait to see what the rest of the year brings!

Emily Mason
Brain Blast/Community Outreach

Community outreach is a key mission of the Vanderbilt Brain Institute (VBI). Throughout the year, the institute hosts events for all ages, including interactive learning for children, seminars, and lectures. These events are organized in large part by the Neuroscience Student Organization Outreach Coordinators LeAnne Kurela and Chelsea Snarrenberg, who were nominated and elected by fellow students in the neuroscience graduate program. Why the emphasis on outreach? “The VBI neuroscience program is committed to training some of the country’s best up-and-coming scientists,” explained Kurela. “I think that the only way to be a good scientist is to pass on the knowledge into the community and to the people that we are really doing the research for - also, ultimately the people who will be funding the work we do.”

In 2014, the VBI presented the first annual Music and the Mind symposium. This event was developed to explore novel connections between neuroscience, psychology, and music. As anticipated, this idea was incredibly popular with members of the Music City community, especially with the inclusion of critically acclaimed singer-songwriter Ben Folds and Daniel Levitin, author of This Is Your Brain on Music in the program schedule. Vanderbilt faculty members Marianna Ploger and Dr. David Zald were featured as discussants, addressing music perception and psychology, respectively.

In the summer of 2015, The Conte Center, a VBI-affiliate, partnered with Room in the Inn to provide science-based lectures for the Nashville residents served by this community shelter. Each week, between 10 and 30 students gather to learn from and discuss with Vanderbilt science and engineering graduate students, including those from our neuroscience community. As an incentive to engage in these learning opportunities, Room in the Inn residents can earn credits for purchasing items at the shelter. This lecture series is noteworthy because it allows neuroscience graduate students to share their science with a lesser-reached part of the Nashville community. Gwynne Davis, Conte Center Scholar and co-organizer of these events, emphasized that involvement in the ‘Science of Our Lives’ lectures “contributes to a unique experience for everyone involved.”

Among the most popular outreach events, for graduate students and the community alike, is Brain Blast. This event is the highlight of the yearly VBI celebration of Brain Awareness Month each March, and was attended by nearly 420 families in 2015. Brain Blast is free, primarily targeted toward young children and adolescents, and seeks to engage the public in hands-on learning and raise awareness about the brain in health and disease. Graduate students and research staff from VBI-affiliated labs, including neuroscience graduate students, are in charge of facilitating interactive booths. This year, booths ranged from activities such as ‘building’ neurons and extracting DNA from strawberries to visualizing brain waves and exploring perceptual illusions.

In addition to this child-targeted event, Brain Awareness month is also a time for VBI-hosted community lectures, which “are one way that we can get ourselves and our work out of the ivory tower that is the university and into the world, where it matters,” as explained by Kurela. This year’s lectures were sure to appeal to a variety of interests. Dr. Mary Phillips from the University of Pittsburgh spoke on her research using neuroimaging to explore mood and behavior disorders in children and adolescents. Dr. Kenneth Catania, a member of Vanderbilt’s neuroscience faculty, shared from his wealth of knowledge about the brains and behaviors of unusual predators.

Kathryn E. Unruh

Child holding human brain at Brain Blast 2015
RESEARCH HIGHLIGHTS

Friendship or Betrayal? Playing the Prisoner’s Dilemma with ASD
Kathryn E. Unruh

Engaging in socially appropriate play behavior is a fundamental milestone in childhood. Play is critical in facilitating development of many skills, including cognitive and social skills. Unlike their typically developing peers, children with Autism Spectrum Disorder (ASD) show limited engagement in play behaviors. Not surprisingly, these children often show significant social cognitive impairments. For example, it may be difficult for an individual with ASD to interpret the behavior of others or empathize and predict how others may think, feel, or act.

Studying the neural basis of play behaviors can provide insight into social salience networks. The prisoner’s dilemma (PD) is a well-established paradigm that can be used together with functional imaging (fMRI) to study activation of brain regions associated with social cognition. In this task, participants can choose to either cooperate or defect with their playing partner; partner pairings vary between a human and a computer. Thus, this task elicits states associated with either positive social outcomes, such as friendship and trust (cooperation) or negative feelings, such as anger or contempt (defect). Accordingly, recruitment of reward-, learning-, and affective-related regions is seen in typical populations.

Edmiston and colleagues implemented a modified version of this task in a sample of 42 children (range = 8 – 12 years; mean age = 10.14 years) with ASD and typical development (TYP). At the beginning of each trial, participants viewed a 2x2 matrix for cooperation and defection, with associated monetary value for each possible outcome. During half of the trials, participants were told they were playing against a computer, otherwise they were playing against a same-gender peer they had met during a previous part of the experiment. Based on what is known about social deficits in ASD, the authors hypothesized similar behavioral performance across groups, but altered neural response. ASD participants were expected to show altered activation in regions that process social salience, including the amygdala, insula, and temporal parietal junction (TPJ).

As hypothesized, behavior performance across groups did not differ. Both groups chose to cooperate in approximately one-third of both human and computer trials and defect in approximately two-thirds of trials. Such a pattern of increased defecting is typical of this age group, as reported in previous studies; however, this pattern resulted in cooperation data being underpowered to detect significant results for region of interest (ROI) fMRI analysis.

Typically developing children showed significantly greater activation of the TPJ, insula, and amygdala during human player defection trials than ASD participants. TPJ activation may be recruited when predicting social behavior of others. Insular function has been previously associated with self-awareness; both the insula and amygdala have been associated with interpersonal and negative emotional experiences. Together, these data suggest social interactions fail to activate regions necessary for detection of salient events in individuals with ASD. Interestingly, individuals with ASD have been shown to have robust insular activation in response to objects related to restricted interests, suggesting this region of the salience network may be activated in response to nonsocial sources of stimulation, rather than social. However, due to data limitations, analyses of the current study were not able to approximate neural responses to nonsocial motivation (computer cooperation). Previous studies would benefit from further comparison between social and nonsocial sources of stimulation to determine if alternate patterns of salience network activation may map onto specific aspects of ASD symptomology.


The Influence of GABRG2 Mutations on GABA<sub>A</sub> Receptor Assembly and Trafficking.
Franklin D. Echevarria

Epilepsy is a neurological disease characterized by the presence of seizures that with the majority of cases have no immediate underlying cause. However, about 30% of epilepsy cases are due to genetic mutations in ion channels, including GABA<sub>A</sub> receptors, which mediate inhibitory activity in the CNS. GABA<sub>A</sub> receptors are made up of five subunits (two α, two β, one γ), with mutations in the γ2 associated gene GABRG2 being the most prevalent among genetic cases of epilepsy. Although not fully understood, past research implicates these mutations in altering recep-
tor function through deficits in receptor biogenesis and/or channel function. Three characterized mutations in the GABRG2 gene—R82Q, P83S and N79S—all occur in the same structural loop of the N-terminus of the γ2 subunit. However, it is not fully known whether these individual mutations affect GABA_A receptor assembly and trafficking similarly.

To answer this question, Vanderbilt neuroscience graduate student Xuan Huang transfected wildtype and mutated GABRG2 cDNA constructs associated with the three mutations and wildtype α1/β2 cDNA constructs in both HEK293T cells and rat cortical neurons. Following transfection, a number of biochemical and functional assays were completed to look at GABA_A receptor function and assembly.

Using flow cytometry, surface biotinylation and immunocytochemistry to study surface expression of WT and mutated γ2 subunits, Huang et. al found that transfection of both the R82Q and P83S γ2 mutations led to decreased surface expression. However, this was not seen in the N79S γ2 mutation. Interestingly, like most mutations that impair protein trafficking to the cell surface, the authors found that lowering the temperature slightly, yet significantly rescued the effect the R82Q and P83S γ2 mutations had on surface expression. In regards to the effect of these mutations on the capability of these receptors to successfully pass a current, functional analysis using whole cell voltage clamp showed that all three mutations led to deficits in GABA_A receptor current, although the deficits were more prevalent in the R82Q and P83S γ2 mutations.

Next, the authors set out to characterize the mechanism behind the opposing effects on surface expression and overall GABA_A receptor current between the R82Q and P83S γ2 mutations and the N79S γ2 mutation. Using western blotting, the authors found that the R82Q and P83S γ2 mutations, but not the N79S γ2 mutation, prevented the γ2 subunit from effectively joining the α1/β2 subunits to form viable receptors and were subsequently trapped and degraded in the ER.

Taken together, Huang et. al demonstrated that the R82Q and P83S γ2 mutations lead to decreased cell surface expression of the γ2 subunit and decreased interaction with the α1/β2 subunits. Functionally, this leads to a deficit in the GABA_A receptor current within the cell. Interestingly, these effects were not as prevalent in the N79S γ2 mutation. Using computer modeling, the authors suggest that the R82Q and P83S γ2 mutations, but not the N79S γ2 mutation are at the γ2/β2 interface, which may explain why the former mutations are more detrimental.


Clock Neuron Firing Rate is Both Input and Output of the Brain’s Circadian Clock
Elaine Ritter

The coordination of behavior with the light cycle occurs in the suprachiasmatic nucleus (SCN), otherwise known as the brain’s circadian clock. Neurons comprising the SCN operate under their own “molecular clocks”, or cyclical patterns of gene expression, and endogenous firing rhythms. The orchestrated synchrony of gene expression and electrical activity are absolutely essential for normal metabolism, homeostatic processes, cognitive function, and ultimately behavior. Despite decades of intensive study, the interplay between the molecular and electric clocks and animal behavior remain elusive – due in part to the difficulty in targeting and manipulating clock neuron activity in awake animals without the use of confounding reagents.

Jeff Jones and Michael Tackenberg, both Neuroscience graduate students in Dr. Doug McMahon’s lab, took an innovative approach to study the electrical component of the circadian clock. Optogenetic techniques allow selective manipulation of firing activity by exposure of rhodopsin channels expressed in neurons to specific wavelengths of light. The authors used a SCN-neuron specific gene to drive expression of two optogenetic transgenes, ChR2 and NpHR, that allow activation and suppression (respectively) of neuronal firing. To interpret the molecular consequences of altering firing rates, they crossed a clock gene luciferase reporter mouse line with their optogenetic transgenic animals.

In brain slice cultures, Jones et al. discovered disturbances in endogenous molecular circadian rhythms when neuronal firing rate was optogenetically increased or decreased. One might expect that blocking the generation of action potentials with a sodium channel blocker would diminish the effects of optogenetic stimulation on circadian rhythmicity, which is indeed what the authors discovered. Interestingly, Jones et al. also report that disrupting signaling of a neuropeptide, VIP, also prevents disturbance of the molecular clock when neuronal firing rate is increased.
One of the strengths of the authors’ use of transgenic mouse lines is the ability to determine the effects of optogenetic manipulation in vivo. In adult mice supplied with fiber optics implanted into the SCN, increasing SCN neuronal firing rate repeatedly led to establishment of a new behavioral circadian rhythm, similar to the effects of traditional light entrainment. Once entrained to the stimulus evoking faster firing, the mice continued to exhibit increased locomotor activity even after optogenetic stimulation had ended. Control animals exposed to the same stimulus via fiber optic SCN implants, but without channel rhodopsin expression in SCN neurons, did not display any alteration in locomotor activity.

The clever use of optogenetic techniques coupled with fluorescent reporters of gene expression, pharmacology, and behavioral assays revealed a novel feature of the SCN electrical clock. Researchers previously thought that circadian patterns of action potentials in the SCN were the result of the endogenous molecular clocks, and these two components together ultimately drive circadian behavior. The results reported by the McMahon lab demonstrate that interfering with SCN electrical cycles alone leads to long-term changes in circadian rhythms – ultimately, the electrical cycles themselves are not merely an output of the circadian clock, but functional to regulate rhythmicity.


The Time Traveling Brain
Kathryn E. Unruh

The phenomenon that is time travel is a common experience; even just thinking back to a time when you have experienced remembering the past in itself a moment of mental time travel. Although the hippocampus is the brain region most commonly identified with memory, many regions in the medial temporal lobe (MTL) are thought to underlie ‘remembering.’ Previous research has put forth hypothesis regarding the roles of specific regions. The posterior MTL, which includes the parahippocampal region, is thought to be sensitive to the temporal and contextual aspects of memory. Alternatively, the anterior MTL and perirhinal cortex has been shown to be active when remembering item-specific information, such as objects and their features. Information from these regions is thought to converge upon the hippocampus, thus allowing binding of objects/items in a temporal sequence.

It has remained undetermined what cognitive processes are supported by the neural responses in these regions. Kragel and colleagues (2015) related this question specifically to memory search, by comparing computational memory models to neural responses during an item-retrieval task. In the task, participants first viewed a sequence of words one at a time. Then they were asked to recall the items, in no specific order, while functional imaging (fMRI) was used to record neural responses. The task was designed so brain activity during recall and termination of recall (when participants ceased to remember any more items) could be measured.

Two cognitive processes interact during these recall events: temporal reinstatement (TS) and retrieval success (RS). When an item is remembered, it is likely that items encoded in close temporal proximity to the recalled item will also be recalled. To put it simply, if you successfully recall item 5 in the list, it is more likely that one of the next items you recall will be 3, 4, 6, or 7. Retrieval success is the neural activity that codes for the balance between successful recall (continuing to remember more items) and termination (not being able to remember any more items). Computational models were developed to predict neural activity based on these cognitive operations. These probabilities were then compared to neural activity from 20 healthy adults during the retrieval task; fit statistics were calculated to determine how well each model predicted activity in each of the previously described MTL regions.

Results revealed differential predictive power of models depending on brain region. When increased activity was seen the parahippocampal cortex and temporal fusiform cortex during a recall event, there was a significantly greater chance that the next item would be from an item in close proximity during stimulus presentation. In contrast, perirhinal cortical activity did not predict these patterns; instead, it varied with recall success and termination. Together, these findings support the hypothesis that anterior and posterior regions of the MTL contribute to different aspects of time travel, with anterior regions supporting retrieval success cognitive operations and posterior contributing to the temporal reinstatement feature of recall. Further, this study demonstrates an elegant use of computational models in coordination with brain activity, and provides proof of concept for using these methods to further assess the neural basis of cognitive constructs.
Hyper and Distracted: A New Mutant Mouse Model of ADHD
Elaine Ritter

Of all pediatric neuropsychiatric disorders, Attention-Deficit/Hyperactivity Disorder (ADHD) is by far the most commonly diagnosed. While initial diagnoses occur less frequently in adults, the majority of ADHD children retain symptoms throughout life. Similar to many other disorders, there are no known biomarkers of ADHD – however, a male-dominant sex bias in diagnoses worldwide suggests a genetic component in the development of attention deficit. The most successful pharmacological treatments for ADHD target the dopaminergic system, specifically the activity of the dopamine transporter (DAT).

DAT global knockout mice exhibit classic ADHD symptoms, such as dramatic hyperactivity, but also suffer severe developmental delay and inability to thrive. Additionally, complete loss of function of DAT in humans typically results in disease symptoms more similar to Parkinson’s disease. Researchers of ADHD need a construct- and face-valid mouse model of ADHD to facilitate studies of dopaminergic signaling in ADHD and discovery novel therapeutic options. A point mutation in the DAT gene that converts amino acid 559 to Valine from Alanine has been recently described in human patients with ADHD, which led members of Dr. Randy Blakely’s lab to generate a novel DAT Val559 mutant mouse line.

Extensive characterization of the DAT Val559 mutant cells demonstrated an anomalous efflux of dopamine through the transporter, indicating an important and conserved role of this amino acid in the function of the transporter. The DAT Val559 mutant mice did not display any overt defects in growth and development, reflexes, or general motor function – however, homozygous mutants were quicker in fall in a wire hanging assay compared to wildtype and heterozygous animals. Given the biochemical findings noted by the authors, the DAT Val559 mutant line is a valuable tool for further study of ADHD mechanisms.


Cerebral blood volume in the hippocampus as a biomarker for schizophrenia
Franklin D. Echevarria

Schizophrenia is a debilitating mental disorder that is associated with abnormal social behavior and the inability to differentiate between reality and fiction. Like most mental disorders, Schizophrenia is diagnosed though reported experiences of an affected individual and behaviors observed by others. Unfortunately, these criteria are subjective and can lead to misdiagnosis as many mental disorders can present with similar symptoms. Therefore, current research is focused on finding an objective biomarker to assist with diagnosis.

Through multiple fMRI- based imaging techniques, research over the past two decades points to hippocampal activity as a target for such a biomarker. More recent studies mapping cerebral blood volume (CBV), a marker of brain activity using fMRI, suggests an increase in hippocampal CBV in those diagnosed with schizophrenia. However, the results of these studies suggest increased CBV in either the CA1 or the CA2/3 sub-region of the hippocampus, findings that may be due to different experimental methodologies.

To settle the debate on whether CBV is elevated in the CA1 or CA2/3 regions of the hippocampus, senior author Pratik Talati, a Vanderbilt neuroscience MSTP student, his advisor Dr. Stephan Heckers MD and their colleagues performed T1-weighted steady state MRI in 15 patients with schizophrenia and 15 healthy controls. Talati et. al acquired a series of coronal images perpendicular to the long axis of the hippocampus, from which the series from each subjected tested was numbered and aligned using the uncus as a landmark. To map and compare CBV in the CA1 and CA2/3 sub-regions, the authors used a contrast agent to highlight tissue and CBV was calculated as a difference between post and pre contrast signal.

When analyzing the CBV of hippocampal subfields CA1 and CA2/3 in all the slices acquired, Pratik et. al found a strong trend (p=0.06) of elevated CBV in the CA1 of schizophrenic patients and a non-significant decrease in CA2/3 CBV in patients compared to healthy controls. Despite the non-significance of the CA1 increase among schizophrenic patients, these results combined lead...
to a significant diagnosis by subfield interaction significant diagnosis by subfield interaction. Taken as a whole, these results support the hypothesis of increased CBV in the CA1 subfield of the hippocampus in individuals diagnosed with schizophrenia.

Despite these results suggesting that CA1 CBV could be used as a biomarker for schizophrenia, the authors stress that these results are preliminary. Despite having sample sizes similar to other studies of this nature, larger cohorts will provide a more detailed picture of the relationship between CA1 CBV and schizophrenia. Additionally, the authors suggest that future studies should focus on the relationship between hippocampal hyperactivity and the generation of schizophrenic symptoms, which may help with early detection of schizophrenia and the prevention of psychotic episodes.


**RESEARCH BRIEFS**

**Neurocircuitry underlying risk and resilience to social anxiety disorder**

*Kathryn E. Unruh*

Temperament in childhood has been shown to be related to the later development of a variety of psychiatric disorders. Children who display an inhibited temperament throughout childhood and into adolescence have a significantly higher risk of developing social anxiety disorder than those with non-inhibited temperaments. Not surprisingly, individuals in these groups show similar behavioral and brain response patterns, especially when anticipating social interactions. In spite of these similarities, some children with inhibited temperament don’t go on to develop social anxiety disorder.

Recent work in the Blackford lab has shown that increased activation of prefrontal cortex and decreased activation of the amygdala may underlie the ability to regulate emotions when anticipating an aversive event. In this study, Clauss and colleagues hypothesized these neural patterns may also characterize inhibited individuals who do not develop social anxiety disorder, suggesting resilience to psychopathology. Inhibited and uninhibited individuals were scored on measures of social anxiety and brain responses were recorded in response to fearful and neutral faces.

Activation of several frontal regions differentiated inhibited and uninhibited participant group; however, greater activation of the anterior cingulate cortex was related to both lower social anxiety and enhanced emotional regulation skills. Importantly, this data suggests that engaging areas of cognitive control during social anticipation may actually be protective against risk for social anxiety disorder. This data supports theories of cognitive behavior therapy, which are commonly used in the treatment of anxiety disorders, and emphasize the importance of investigating brain-based underpinnings of therapeutic approaches to improve treatment response.


**A Type II Diabetes Treatment Holds Promise for Drug Addiction Therapy**

*Elaine Ritter*

Drugs of abuse and dependence reinforce reward-seeking behavior at least in part by affecting the midbrain dopaminergic circuitry found in the nucleus accumbens (NAc) and ventral tegmental area (VTA). Logically, numerous research efforts focus on identifying therapeutic candidates that interact with the dopamine system – but one might not expect a neuropeptide receptor in the primarily expressed in the gut to be a prime target for drug dependence therapy.

Glucagon-like peptide receptor 1 (GLP-1) is activated in the intestine in response to food intake, and GLP-1 agonists effectively treat Type II Diabetes. However, GLP-1 is also expressed in the neurons of the nucleus of the solitary tract and directly project to the NAc and VTA. India Reddy, Gregg Stanwood, and Aurelio Galli collaborated with researchers in Denmark to study how a GLP-1 agonist, Exendin-4, might affect drug-seeking behavior in rodents.

The authors found a reduction of the reinforc-
ing effects of cocaine in mice exposed to Exendin-4, even in the context of chronic cocaine use. Exendin-4 did not affect self-administration of the vehicle control, indicating specific action in reward reinforcement. The findings reported provide incentive to further explore GLP-1 receptors as an avenue for drug addiction therapy, especially given that GLP-1 receptor agonists are already used in the clinic.


Use of Resting-State Functional Connectivity to show CNS differences in typically developing individuals and those with neurodevelopmental disorders.

Franklin D. Echevarria

Neurodevelopmental disorder is a broad term that encompasses a wide range of CNS deficits. To better understand of the functional architecture between typically developing (TD) controls and individuals with either down syndrome (DS) and Williams syndrome (WS), Jennifer Vega and colleagues utilized a functional imaging technique known as resting-state functional connectivity (rsFC) analysis to test for in between- and within-network blood oxygen level-dependent signal differences in seven functional networks (default mode, dorsal attention, ventral attention, frontoparietal, limbic and visual).

Compared to TD controls, the authors found those with DS displayed significantly increased connectivity in 6 brain regions. However, when compared to individuals with WS, no significant difference was noted. Interestingly, when comparing WS individuals with TD controls, only 1 brain region in WS individuals showed significantly increased brain connectivity. Despite differences in between network connectivity, no differences were noted within- network connectivity in DS individuals. Overall, these results replicate a previous report suggesting increased connectivity between DS individuals and TD controls. Additionally, these results suggest possible differences in brain connectivity between different types of neurodevelopmental disorders when compared to TD controls.

On the Cover

The artwork used for the VRN cover image symbolizes a scientist’s curiosity, passion, and drive to understand the mysteries of the brain in the field of neuroscience from different perspectives. These layers of observations and insights made by a scientist may involve molecular and cellular approaches to comprehending normal brain function and abnormalities, neuronal systems and global brain function, or both. Moreover, the artwork, particularly the sketches of the mouse and man, emphasizes the challenges of using animal models to predict human responses or outcomes and the need to invent scientific tools that can enhance the predictive power of such animal models.

This artwork was inspired by the dedication of current and future scientists: (1) to advance the field of neuroscience research through scientific inquiry; and (2) to thoroughly examine the brain with the aim of alleviating diseases and disorders of the brain which plague everyday citizens across the world.

As a neuroscience doctoral student in Dr. Sachin Patel’s laboratory, my dissertation research project focuses on the role of the endocannabinoid (eCB) system in stress-induced maladaptations in the mouse brain, specifically the amygdala. It will test the hypothesis that substrate-selective inhibitors of cyclooxygenase-2 (SSCIs) have anxiolytic potential by augmenting eCB levels in the brains of animals after stress exposure using ex vivo field electrophysiological recordings, behavioral assays of anxiety, and biochemical techniques. This research investigation will provide valuable information to further the discovery of promising pharmacologic agents that can prevent or treat anxiety, stressor-related, and trauma-related disorders, such as acute stress disorder and post-traumatic stress disorder.

Joyonna Gamble-George
Introduction

Stress is a major environmental risk factor for psychiatric disorders including major depression (MD) and anxiety disorders (ADs). Although these disorders are leading causes of disability and represent large economic and societal burdens in terms of health care costs and reduced productivity, the neurobiological mechanisms by which stress is translated into psychopathology are poorly understood. First-line treatments for MD and ADs reduce symptoms by augmenting monoaminergic transmission but only have long-term efficacy ranging from 40-60%.

Neuroimaging studies have correlated MD and ADs with amygdala hyperactivity. The amygdala is well-established as a key modulator of neuroendocrine and behavioral responses to stressful stimuli. Furthermore, in rodents, stress increases excitatory drive to the basolateral nucleus (BLA), one of the subnuclei of the amygdala. These and other data suggest that reducing glutamatergic drive to the amygdala may mitigate the adverse effects of stress.

Mounting evidence suggests a role for bioactive lipids like endogenous cannabinoids in the modulation of stress, mood, and related pathologies. Endogenous cannabinoid (eCB) signaling inhibits glutamatergic inputs to the amygdala, indicating that manipulation of this system may be able to reduce stress-related amygdala hyperactivity, and suggesting this as a novel treatment approach for MD and ADs.

The eCB system is composed of two cannabinoid receptors (CB1 and CB2), two primary ligands, N-arachidonylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), and their synthesizing and degrading enzymes. The cannabinoid receptors were originally discovered as the receptors through which the primary psychoactive component of cannabis, Δ9-tetrahydrocannabinol (THC), exerts its effects. CB1 is more highly expressed in neurons and has been implicated in regulating a host of synaptic and behavioral processes. Interestingly, the most commonly reported reasons for using cannabis are to relieve anxiety and elevate mood which suggests that increasing cannabinoid signaling may be a particularly effective strategy for reducing anxiety and depression. Unfortunately, direct cannabinoid agonists elicit acute, intense anxiety responses in a subset of people, impair short and long term memory formation, and chronic use causes CB1 desensitization. An alternate pharmacological approach is to manipulate the endogenous cannabinoid system. Indeed, augmenting endogenous cannabinoid (eCB) signaling by inhibiting eCB degradation reduces stress-induced anxiety- and depressive-like behaviors in rodents. Most translational studies have focused on the role of the first discovered eCB ligand, AEA, in the pathology of stress-related psychiatric disorders and animal studies have convincingly demonstrated that elevating levels of AEA in the central nervous system (CNS) can mitigate some of the physiological and behavioral effects of stress. However, relatively little is known about the role of 2-AG, the most abundant eCB ligand, in stress-related psychiatric disorders. Here I will review what is known about 2-AG metabolism, stress-related changes in 2-AG signaling in the amygdala, and the current evidence for 2-AG’s involvement in stress-related neuropsychiatric disorders with a focus on key open questions and avenues of investigation.

2-AG Metabolism: a Nexus of Bioactive Lipids
Two additional serine hydrolases, ABHD6 and ABHD12, although most evidence indicates that although further investigation on this, studies have shown that SAG is suggested to be the major. Mice lacking DAGL α are increasingly impaired 2-AG signaling while mice lacking DAGLβ have less than a 50% reduction in brain 2-AG content and no impairment in 2-AG mediated synaptic signaling. These data indicate that DAGLα is, perhaps exclusively, responsible for 2-AG synthesis in the context of retrograde synaptic signaling.

Interestingly, reduced activity of DAGLα; mutations in ABHD12, which have as such, further investigation of the. These data indicate that DAGL α while DAGLβ and the negative.

Two diacylglycerol lipase isoforms (DAGL α and β) have been cloned, differing primarily by DAGLα’s longer C-terminal tail which likely facilitates interactions with scaffolding proteins. In mature neurons, DAGLα is prominently expressed in dendrites immediately adjacent to CB1 expressing axon terminals while DAGLβ is expressed more broadly in peripheral tissues. Mice lacking DAGLα produce up to 80% less 2-AG in the CNS and exhibit extremely impaired 2-AG signaling while mice lacking DAGLβ have less than a 50% reduction in brain 2-AG content and no impairment in 2-AG mediated synaptic signaling. These data indicate that DAGLα is, perhaps exclusively, responsible for 2-AG synthesis in the context of retrograde synaptic signaling.

Altogether, the evidence strongly suggests that the PLCα-DAGLα pathway plays an important role in the biosynthesis of 2-AG at synapses. However, the effect of PLC and DAGL inhibitors on 2-AG mediated synaptic signaling is not always consistent indicating that regulation of on demand biosynthesis of synaptic 2-AG is not likely to be so simple. As such, further investigation of the other proposed biosynthetic pathways, which have been largely ignored in recent years, is necessary. Currently available DAGLα inhibitors, are not sufficiently specific to conclusively indicate its involvement (or lack thereof) in synaptic processes and global knockouts of DAGL genes are likely to have developmental adaptations which could mask normal physiological roles of the DAGLs. The development and use of more specific DAGLα inhibitors or of a conditional DAGLα knockout would be a huge step forward in conclusively establishing the requirement for DAGLα in 2-AG synaptic signaling and stress-induced alterations of 2-AG signaling capacity, which will be discussed in more detail below.

2-AG Degradation

While there are also at least two biochemical pathways implicated in 2-AG degradation, studies have shown that 98% of 2-AG is hydrolyzed into arachidonic acid and glycerol by serine hydrolases, with the majority of this activity (~85%) in the CNS attributed to monoacylglycerol lipase (MAGL). Two additional serine hydrolases, ABHD6 and ABHD12, account for a further 13% of 2-AG hydrolysis in rat brain membrane preparations. Interestingly, reduced activity of each of these serine hydrolases has distinct effects: ABHD6 inhibition reduces epileptic activity and the negative impact of traumatic brain injury; mutations in ABHD12 lead to a neurodegenerative disease called PHARC; and MAGL inhibition, which is best studied, has a host of effects including antidepressant and anxiolytic actions which will be discussed later in this review.

Most evidence indicates that MAGL is the primary 2-AG degrading enzyme. RNAi-mediated knockdown of MAGL in HeLa cells enhances the accumulation of 2-AG.
following application of ionomycin which stimulates the production of 2-AG by elevating Ca2+ intracellularly\textsuperscript{76}. Additionally, immunodepletion of MAGL from rat brain homogenate results in a significant reduction in 2-AG hydrolysis\textsuperscript{76}. Whereas DAGLα is expressed post-synaptically, MAGL is co-expressed with CB1 in presynaptic compartments allowing for tight coupling of synthesis, retrograde signaling, and degradation of 2-AG\textsuperscript{92}. Genetic deletion and chronic high-dose MAGL inhibition lead to extreme elevations in 2-AG and CB1 receptor desensitization\textsuperscript{83,84}, but chronic low-dose MAGL inhibition maintains anxiolytic and antidepressant efficacy in mice without down-regulating CB1\textsuperscript{29,81}, supporting its potential utility as a novel approach for treating MD and ADs.

**Amygdala 2-AG Signaling: Effects of Chronic Stress Exposure**

CB1 activation reduces presynaptic neurotransmitter release through a variety of mechanisms. 2-AG is a full agonist at CB1 receptors which are Gi/o coupled GPCRs broadly expressed on axon terminals of both GABAergic and glutamatergic neurons throughout the brain\textsuperscript{95}. CB1 activation affects a variety of intracellular signaling cascades: it inhibits adenyl cyclase thereby reducing intracellular cAMP levels and PKA activity\textsuperscript{86}, stimulates intracellular kinases\textsuperscript{87,90}, inhibits N- and P/Q-type Ca2+ channels\textsuperscript{93,92}, and activates inwardly rectifying K+ channels\textsuperscript{93,94}. The overall effect of CB1 activation is typically a reduction in presynaptic neurotransmitter release probability. This modulation of synaptic efficacy is a type of inhibitory synaptic plasticity and can last from seconds to minutes (short-term depression) to over an hour (long-term depression).

Chronic stress exposure is widely used to elicit anxiety- and depressive-like behaviors in mice. Given that repeated life stress is such a strong environmental risk factor for the development of MD and ADs, it seems likely that mechanisms underlying stress-related behavioral dysregulation in mice could also be at play in these pathological conditions in humans. Chronic homotypic stress exposure elicits behavioral and neuroendocrine habituation, meaning that after repeated daily exposure to the same stressor, mice exhibit fewer escape attempts and much less corticosterone release than during the first stress exposure\textsuperscript{95}. Concurrent with this gradual habituation, 2-AG in the amygdala increases progressively and 2-AG mediated plasticity is enhanced. The duration of depolarization induced suppression of inhibition (DSI), a 2-AG mediated form of short-term depression of GABAergic transmission, is increased\textsuperscript{97}. Sumislawski et al. 2011 also demonstrated that chronic homotypic stress increases amygdala 2-AG and additionally found that chronic stress gates the induction of long-term depression of GABA transmission (LTDi) by low-frequency stimulation in the BLA\textsuperscript{95}. It was further suggested that this elevation of 2-AG could be due to a reduction of MAGL, although another study using the same stress protocol showed no change in MAGL activity in amygdala homogenates\textsuperscript{96}. Importantly, it has been suggested that progressively elevated amygdala 2-AG and alterations in 2-AG mediated plasticity play a role in producing the behavioral and neuroendocrine habituation that takes place during chronic homotypic stress\textsuperscript{98}, acting as a sort of stress-buffering mechanism. If 2-AG signaling acts as a natural buffer against stress effects, targeting this system could be an extremely effective approach for treating stress-related pathology.

The amygdala is one of the key limbic structures whose activity promotes hypothalamic-pituitary-adrenal (HPA) axis activation\textsuperscript{99}. Local administration of a CB1 agonist into the BLA reduces activation of the HPA stress axis\textsuperscript{100,101} while CB1 antagonism increases neuronal activation in the BLA and increases corticosterone release in response to stress exposure\textsuperscript{89,102}. As discussed in the 2-AG synthesis section of this review, 2-AG production and signaling is typically activity-dependent in that it usually occurs as a result of postsynaptic depolarization, Gq/11-coupled receptor activation, or both. In addition to elevating amygdala 2-AG\textsuperscript{95,97,103}, chronic restraint enhances Fos immunoreactivity of BLA pyramidal neurons, but not interneurons\textsuperscript{104}. This suggests a specific increase in glutamatergic activity\textsuperscript{104} of the BLA following stress; however, the majority of synaptic studies have focused on 2-AG-mediated suppression of GABAergic signaling in the BLA following stress exposure as indicated by the DSI and LTD\textsubscript{i} examples above. The impact of stress on 2-AG mediated suppression of glutamatergic signaling in the BLA has not been examined. Further indicating the importance of investigating eCB-mediated suppression of glutamatergic signaling in the context of stress, conditional CB1 deletion from forebrain glutamatergic neurons enhances anxiety responses to intense stress as effectively as CB1 deletion from all CaMKII expressing projection neurons\textsuperscript{105} suggesting that it is CB1 on glutamatergic terminals that is critical for the regulation of stress and anxiety responses. To truly test the ability of 2-AG signaling to modulate stress-related glutamatergic hyperactivation of the amygdala and stress habituation, region-specific DAGLα deletion is required.

**2-AG in Major Depression and Anxiety Disorders**

In addition to inducing changes in eCB-mediated plasticity in the amygdala, chronic stress induces multiple
Acute pharmacological JZL-184 treatment is suggesting that 52, 64, 114. DAGL. Given that chronic unpredictable stress and clinical trials. Direct CB1 agonists are. For example, in another anxiety test, the elevated and chronic heterotypic stress. It has been shown that adult neurogenesis is. JZL-184 treatment, suggesting either that this phenomenon. Interestingly, the same phenomenon is seen in the elevated plus maze when mice are treated with an inhibitor of the primary AEA degrading enzyme, fatty acid amide hydrolase (FAAH)35 suggesting either that this aversion-enhanced responsivity is downstream of the eCB ligand, or that a common mechanism is able to regulate the signaling of both eCB ligands. Enhancing 2-AG signaling during chronic stress exposure reduces the anxiogenic and depressant effects of both chronic homotypic and chronic heterotypic stress30. Indeed, Zhong et al. 2014 show that chronic JZL-184 treatment concurrent with chronic unpredictable stress (CUS) exposure prevents the typical CUS-induced development of anhedonia in the sucrose preference test, immobility in the forced swim test, and anxiety in the novelty induced suppression of feeding test30. Given that chronic unpredictable stress has been shown to reduce hippocampal neurogenesis and dendritic arborization, it would be interesting to test if JZL-184 treatment is able to prevent these changes as well, since they have also been implicated in the pathology of depression and are reduced by typical antidepressants. Interestingly, peripheral 2-AG is significantly reduced in women with MD as compared to healthy controls113 suggesting that impairments in 2-AG may be involved in either susceptibility or pathogenesis of depression, although it is not known how or even if peripheral eCB levels are related to central eCB levels. While this correlation is compelling, there is no causal evidence indicating that specific impairment of 2-AG signaling elicits anxiety or depression. Although DAGLα knockout mice do exist, very little behavioral testing relevant to psychiatric disease has been reported. Instead, the primary focus has been on determining that DAGLα is, in fact, responsible for producing the majority of synaptically relevant 2-AG signaling within the amygdala. 2-AG-mediated suppression of glutamatergic drive into the amygdala may contribute to psychiatric disease like major depression and anxiety disorders. Chronic stress elevates 2-AG content and signaling within the amygdala. 2-AG-mediated suppression of glutamatergic drive into the amygdala may contribute to stress habituation. 2-AG signaling in the amygdala could act as an endogenous stress-buffering mechanism. As such, impaired 2-AG signaling may contribute to the pathogenesis of these stress-related psychiatric disorders. Restoration of 2-AG signaling may constitute an effective treatment for
major depression, anxiety disorders, and other stress-related pathologies.

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Candian Reviews


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Sensory Abnormalities in Autism: Towards a More Unified Characterization

Lauren K. Bryant

The presence of sensory differences is often noted as part of a description of features related to autism spectrum disorder (ASD) and is now included in the DSM-5 diagnostic criteria. In addition to the clear role of sensory integration in the formation of healthy social representations, mounting evidence suggests that alterations in sensory function may also contribute to the language and communication deficits characteristic of ASD. This paper will review auditory and tactile sensory processing in autism. Emphasis is placed upon how psychophysical methods can be used to explore sensory processing in order to better understand the etiology of autism and improve upon therapeutic approaches.

Keywords  autism, sensory, auditory, tactile, somatosensory

Introduction: Sensory Responsiveness in ASD

Autism spectrum disorder (ASD) is a developmental disorder characterized by impaired social interaction and communication and restricted, repetitive patterns of behavior. The recent publication of the Diagnostic and Statistical Manual of Mental Disorders—Fifth Edition now includes sensory disturbances in the diagnostic criteria for autism, defined as: “Hyper- or hypo-reactivity to sensory input or unusual interest in sensory aspects of environment.” Although generally underemphasized relative to deficits in language and social communication, parents and clinicians have noted sensory disturbances since the earliest characterizations of the disorder. Asperger observed: “Over-sensitivity and blatant insensitivity clash with each other…Many children have an abnormally strong dislike of a particular tactile sensation…There is hypersensitivity too against noise.”

Sensory differences in ASD typically extend to multiple systems including vision, hearing and touch. However, this review will focus on those within the auditory and tactile domains. Sound and touch are of particular interest because visual acuity is poor during the first few months of life and infants are likely to rely more heavily on sound and touch to interpret and interact with their environment. Furthermore, vocal cues are frequently accompanied by socially rewarding touch during mother-infant dyadic communication. It is important to review relevant literature which considers how ASD affects socially relevant auditory and tactile interactions because these modalities begin as early as infancy and continue throughout development, and ASD symptoms tend to emerge within the first 3 years of life.

One of the challenges in reviewing the literature on sensory differences in ASD is inconsistency of terminology. Clinicians, therapists and scientists use a wide variety of terms in an attempt to capture an array of sensory abnormalities. For the purposes of this review, atypical sensory responsiveness will be classified into two categories: hyper-responsiveness and hypo-responsiveness. Hyper-responsiveness is broadly defined as an exaggerated behavioral reaction, aversive response (defensiveness) or effort to avoid a sensory stimulus. In contrast, hypo-responsiveness is characterized by the absence of, delayed or diminished response to simple sensory events that typically elicit a response. This review will attempt to cast auditory and tactile behavioral and neurobiological findings within this framework of sensory responsiveness.

Auditory Processing in ASD: Behavioral research

Parent/Caregiver Reports

The most widely used measures of sensory responsiveness in ASD are parent and caregiver reports. The Sensory Profile and the Evaluation of Sensory Processing are two sensory assessments that are not specific to autism but are most frequently used. Using an adapted version of the Sensory Profile called the Short Sensory Profile, Dunn and Tomchek examined differences in sensory processing in 281 children ages 2 to 6 with ASD. Of these children, 77.8% exhibited a definite difference (a score greater than 2 standard deviations from the mean for typically developing (TD) children) in auditory responsiveness. It is important to note that the scenarios presented under auditory filtering encompass what has been defined in this review as both hyper- and hypo-responsiveness. This lack of distinction is common in many studies which use parent reports. To address this concern, Baranek et al. developed a caregiver report assessment called the Sensory Experiences Questionnaire that explicates the nature of hyper- and hypo-responsiveness sensory patterns. Results from this study revealed a high prevalence (69%) of overall sensory symptoms and greater hypo-responsiveness as compared to the TD and developmentally delayed children. More recently, Tan et al. attempted to substantiate second-hand accounts of auditory sensory responsiveness in autism with direct clinical observation using a comprehensive checklist examining auditory abnormalities across four domains. All 156 children with autism presented...
with varied auditory abnormalities, manifested primarily as hypo and hypersensitivity, compared with only 33.3% of language delayed children. Although parent and caregiver questionnaires are generally a very practical way of assessing sensory function in children across the entire autism spectrum, results must be interpreted with caution, as responses infer sensory experience from subjective interpretations of the child’s behaviors. However, these reports are able to provide data based on multiple observations across diverse context. Furthermore, researchers are taking steps to improve upon current assessments. For example, Tavassoli et al. developed the Sensory Perception Quotient to more accurately assess basic perception and avoid items which involve behavioral and affective responses towards sensations. Future work that seeks to differentiate affective aspects of auditory sensory behaviors from sensory perception will be important for parsing sensory abnormalities in ASD.

**Laboratory based measurements**

To validate subjective observations of hypo- and hyper-responsiveness, researchers have examined sensory response behaviors by manipulating various auditory properties. In particular, hyper-responsiveness could be associated with oversensitivity to particular frequencies or volume ranges of sound (e.g. hyperacusis). Using a psychoacoustic test to generate categorical ratings of perceived loudness, Khalifa et al. observed significantly lower loudness discomfort levels to pure tones in individuals with autism compared to TD children. Furthermore, Rosenhall et al. found 18% of children with ASD exhibited loudness discomfort to click stimuli at intensity levels less than 80dBHL, which was absent in the controls. Additional studies demonstrated enhanced auditory perceptual performance in ASD as evidenced by perfect pitch (the ability to label isolated musical notes), discriminate between two very similar pitches and to pick out changes in melodies more easily. Such behavioral findings may be constructive perceptual correlates of heightened auditory sensitivity. Although some researchers have attempted to link auditory sensory behaviors with perceptual performance, additional studies are needed to understand how enhanced auditory perception may more specifically contribute to hyper-responsiveness.

Presently, there is a dearth of experimental research on the perceptual basis of auditory hypo-responsiveness. However, existing studies point to the absence of orientation to auditory stimuli as one example. Although the majority of evidence comes from retrospective analyses of videotapes and parent reports, Dawson et al. found that under controlled laboratory conditions, children with ASD were significantly less likely to orient to sound in general and particularly during the presentation of social stimuli. Importantly, Dawson and colleagues controlled for social content. Yet, much of the research in this area involves complex speech stimuli, which increases cognitive demand beyond basic auditory processing. Such variability in task design clouds conclusions of a relationship between perturbations in auditory processing and atypical responsiveness. Therefore, the present state of conflicting results necessitates further studies that separate basic auditory processing from more complex stimuli.

**Behavioral Variability and Stimulus Complexity**

Observational and experimental research reveals both hypo- and hyper-responsiveness to auditory stimuli in ASD. In some instances, the same individual may exhibit both patterns of sensitivity. Existing research supports the idea that this paradox may lie in the complexity of the stimulus. One such explanation is based on the theory of weak central coherence, which suggests enhanced local processing and impaired global processing. Support for this theory comes from studies that found while children with autism outperform their non-autistic peers in local processing abilities (recognition of single-note changes in melody), the same children showed no improvement over peers in global processing skills, such as perception of changes in key of the entire melody or alteration in melody contour. However, such results are not without contention and researchers continue to build upon existing models. Nevertheless, stimulus complexity and task design are important considerations for the study of auditory processing in ASD.

**Neurological basis of Auditory Processing in ASD**

Atypical auditory processing is possibly related to the unusual behavioral responses often noted in ASD. For this reason, many researchers have sought answers at a neural level. The flow of auditory information processing can be measured through the auditory brainstem response, in which the electrical activity evoked from a series of clicks or tones is recorded using surface electrodes. The auditory brainstem response literature reports varied and contradictory findings. Some studies show no differences in latency or amplitude, while others report prolonged latencies in young children with ASD. Further complicating the matter, a few studies indicate that children with ASD demonstrate typical brainstem responses to clicks, but differences in response to varied pitch and speech sounds with noise. Given that individuals with ASD tend to exhibit normal hearing during audiometric testing, it is likely such incongruence reflects variations in subject populations and stimulus complexity rather than an inherent abnormality at the level of the brainstem.

At the cortical level, auditory sensory processing has traditionally been examined using event-related potentials (ERPs) with electroencephalography (EEG) and magnetoencephalography (MEG). EEG and MEG studies suggest enhanced neural detection of frequency changes in ASD at the pre-attentive level. However, there are also significant differences in later signals often attributed to attentional modulation. More specifically, the P300 ERP component may be of lower amplitude among people with autism. Further support for higher-order differences in atypical auditory processing comes from studies demonstrating...
differential changes in the Blood Oxygenated Level Dependent (BOLD) signal in an oddball detection paradigm during presentation of sounds of varying complexity.\(^{51,52}\) Currently, there is no clear picture of cortical activation during auditory processing in ASD. However, such variation may be influential in deciphering the source of disruption along the chain of auditory processing as it relates to both hypo- and hyper-responsiveness patterns. Recently, Donkers et al. conducted a study in which they correlated measures of sensory responsiveness and aberrant behaviors with multiple ERP components and found that attenuated N2 and P3a amplitudes were associated with greater sensory seeking behaviors.\(^{55}\) Additional studies that attempt to correlate neurological markers of auditory processing with atypical sensory behaviors have the potential to greatly impact therapeutic measures for sensory related issues in ASD.

**Tactile Processing in ASD: Behavioral Research**

**Parent/Caregiver Reports**

Although tactile sensitivity is commonly reported in ASD, it receives far less attention in the neuroscience literature than auditory sensitivity. This is despite the fact that touch is one of the most fundamental senses for social processing and development and moderates much of an infant’s first interactions with the world.\(^{54,55}\) Multiple researchers have reported tactile abnormalities using the SP and SSP and correlated them with other sensory symptoms.\(^{56-58}\) Items that assess tactile sensitivities include: difficulty standing close to others, distress during grooming, unusual reaction to touch, and avoiding going barefoot. Using the scores in the tactile subscales, Kern et al. found significant correlations between touch and the visual and auditory items of the SP.\(^{64}\) Kern and colleagues then reduced the items to those that assessed high and low thresholds and found that items used to evaluate low touch thresholds positively correlated with low threshold auditory items and both low and high threshold visual items. Although this assessment was not experimental, a breakdown of items based on threshold levels begins to address hyper- and hypo-responsiveness in a more direct manner than many previous studies.

Direct observation also complements caregiver reports of tactile responsiveness. One easily manipulated and observable behavior is tactile defensiveness, defined as a tendency to react negatively and emotionally to certain touch situations. This reaction is often attributed to hyper-responsiveness. Creedon and Baranek developed the Tactile Defensiveness and Discrimination Test (TDDT), which allows investigators to identify specific behavioral reactions (e.g., scratching/rubbing the skin, negative facial grimaces, stimulus withdrawal) to a variety of tactile stimuli.\(^ {59}\) In a study examining the relationship between scores on the TDDT and tactile responsiveness, results suggest that tactile defensiveness is best conceptualized on a continuum rather than as discrete category behavior.\(^{60}\) Although a causal relationship with core features of ASD is undetermined, tactile defensiveness is significantly associated with certain kinds of rigid and stereotyped behaviors.\(^{61}\) Consequently, experimentally driven research on tactile defensiveness could prove instrumental for successful remediation of disruptive behaviors.

**Laboratory based measurements**

Many of the psychophysical tactile studies measure thresholds and sensitivity using vibrotactile stimuli. Blakemore et al. used 30 and 200 Hz vibrotactile stimulation in adults with Asperger's syndrome (a form of autism with a lower trait burden) and found significantly lower tactile perception thresholds at 30 Hz, supporting frequency specific hypersensitivity.\(^{62}\) Similarly, Cascio et al. demonstrated hypersensitivity to frequency and thermal thresholds, but not light touch in adults with autism.\(^{53}\) However, O’Riordan et al. reported no differences in the ability to discriminate different tactile stimuli and no significant difference in pressure sensitivities in a small sample of children with ASD.\(^{55}\) Guclu et al. also found no significant difference in vibrotactile thresholds at 40 and 250 Hz in six male children with ASD.\(^{63}\) Interestingly, the same study revealed a high correlation between the data from the tactile and emotional subsets of the questionnaires, albeit a small sample size. Guclu and colleagues interpreted these results as support for the hypothesis that the hyper- and hypo-responsivity to touch may not be a perceptual sensory problem, but instead one of emotional cognition. Such findings have important implications for methodological approaches to sensory assessment in ASD as well as determining the appropriate targets for intervention.

Before trying to determine the source of perceptual differences, there is a need for better psychophysical characterization of such differences. Variability of results in the aforementioned studies could be due to variation in the tactile stimulation used as well as differences in cohort characteristics and stimulus location. Recently, Puts et al. attempted to address these concerns using a battery of different vibrotactile tasks within the same cohorts of typically developing children and those with ASD.\(^ {65}\) The results of this study show significant differences in tactile sensitivity between ASD and TD children on a number of vibrotactile measures, including raised static detection thresholds and an absence of the effect of a dynamically increasing sub-threshold stimulus on static detection threshold in children with ASD. They also showed poorer amplitude discrimination, which could be attributed to hypo-responsiveness, as well as decreased adaptation, potentially reflective of hyper-responsiveness. Future research that broadly assesses tactile sensory function within the same cohort will make it possible to better identify specific neurophysiological and cortical mechanisms underlying abnormal tactile processing in ASD.
Neurological basis of Tactile Processing in ASD

Multiple studies have revealed evidence for aberrant neural processing of tactile information in ASD. Miyazaki et al. report delayed interpeak latency in late somatosensory evoked potentials in autistic children using median nerve stimulation, which they attributed to cortical dysfunction. In addition, MEG studies have shown that children with autism have early differences in somatosensory processing, which may affect later sensory-motor integration. There may also be a developmental component to tactile dysfunction, given that somatosensory mapping in high-functioning adults with autism revealed disrupted cortical representations of their face and hand. Additionally, connectivity analyses suggest local underconnectivity in the somatosensory cortex. Recently, Cascio et al. used functional magnetic resonance imaging in adults with ASD to investigate somatosensory responses to textured surfaces ranging in roughness and pleasantness. Changes in BOLD signal in response to stimulation differed substantially between the groups, with the ASD group exhibiting diminished responses compared to the control group for pleasant and neutral textures. It is difficult to interpret the findings of the aforementioned studies due to dissimilar experimental design. However, diversity in approach and findings may prove valuable. Continued work employing complementary techniques with high spatial and temporal resolution and varied tactile stimulation will contribute to a more intricate understanding of atypical tactile processing in ASD.

Conclusions

There are few sensitive, reliable and valid measures with a strong empirical foundation from which to characterize specific sensory patterns in ASD. Although this review has presented data demonstrating altered responsiveness to auditory and tactile stimuli, empirical evidence that thoroughly characterizes the nature and extent of these changes is absent. Of the studies discussed, the majority reported threshold values without reporting the entire psychometric function (i.e., range of responsiveness) as a result of change in a basic sensory property (e.g., frequency, amplitude). This is critical for operationally defining hypo- and hyper-responsiveness. Psychophysical experiments that measure behavior (e.g., reaction time, accuracy) as a function of changing stimulus intensity is one way to address this issue. Assessing the full range of functioning over a wide range of intensities may provide direction for future work developing novel behavioral and pharmacologic therapies.

Through this brief review, it is apparent that past research has found substantial evidence for atypical processing of auditory and tactile information in ASD, yielding mixed results. One possible reason for the variability in findings is the use of different experimental methods and the co-occurrence of hyper- and hypo-responsiveness that presents a challenge to experimental design. It is also possible that perceptual abnormalities arise not only from differential perceptual function but also from aberrant integration and organization across sensory modalities. An empirical and more comprehensive psychophysical characterization of sensory and multisensory function in ASD is essential to parsing its elusive etiology, and in understanding how alterations in sensory function relate to changes in domains such as language and social communication. Comparisons between psychophysical and caregiver reports of sensory responsiveness could further our understanding of how well observational scales relate to experimentally measured values, and ultimately lead to more informed diagnoses and better targeted treatments.

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Timing is Everything: Multisensory Temporal Coherence

Matthew De Niear

The synthesis of information across the different senses has profound effects on behavior and is essential for the development and maintenance of higher order cognitive processes, including speech and social interactions. The perception of the temporal relationships between sensory signals is fundamental to the development of multisensory coherence. This review describes how the perception of temporal relationships is critical for multisensory processing, remains plastic, and influences the perception of audiovisual communication. The review concludes by briefly describing how increased difficulty in perceiving the temporal relationships of multisensory stimuli may underlie deficits observed in autism.

Keywords Multisensory Processing, Temporal Processing, audiovisual, Autism Spectrum Disorder, Multisensory Speech Perception

The World in Multisensory: Principles

Our world is filled with sights, sounds, tastes, smells, and feelings. The different sensory modalities process a wide variety of environmental stimuli that are integrated to create a unified perceptual experience. The integration of these various sensory stimuli into a single, unified multisensory experience is one of the central elements in the creation of our perceptual reality. This process, termed multisensory integration, refers to the process whereby sensory signals from separate modalities are combined into a single distinct product that cannot be easily isolated from constituent unisensory components. At the level of the single neuron (as demonstrated most prominently in the superior colliculus of the cat, an early model for multisensory integration), multisensory integration is observed as either enhancement or depression of a neural response following presentation of a multimodal stimulus that is significantly different from the neural response elicited separately by the unimodal component stimuli. At the level of an organism, multisensory integration is evidenced by altered behavior and perception, as demonstrated by enhanced target detection, speeded reaction times, and a host of perceptual illusions. Such behavioral and perceptual gains derived from multisensory processing can arise from interactions with the most basic multisensory stimuli (e.g., visual flashes and beeps) to more complex stimuli (e.g., audiovisual speech and biological motion).

Multisensory integration appears to follow three major principles across all levels of analysis. The first principle is the idea of inverse effectiveness, which describes the observation that the largest behavioral gains or alterations in neural activity from multisensory integration occur when the component unisensory elements are minimally effective in eliciting a behavioral or neural response. As the salience of the component unisensory elements increases, the relative effects due to multisensory integration diminish. The second principle, the spatial rule, states that the greatest multisensory enhancement occurs when stimuli are increasingly proximate to each other in space. The third and final principle of multisensory integration and the principle that is particularly relevant to this review is the temporal rule. The temporal rule posits that the largest multisensory enhancements typically occur when paired stimuli are close together in time.

Perceiving Temporal Synchrony Between the Senses

Temporal coincidence has been described as the most important stimulus property that determines whether an organism will perceive separate sensory signals as the same environmental event. While spatial, semantic, and other stimulus properties that interact with temporal properties may affect whether the organism ascribes differing sensory properties as belonging to the same multisensory object, the synchronous occurrence of environmental stimuli is one sensory signal property that allows an organism to reliably associate signals detected by different modalities as originating from the same event. Such a concept has a great deal of face and ethological validity, since energies (e.g., light and sound) that arise at the same time are likely a result of the same object or event. Likewise, asynchronous occurrence of sensory stimuli allows for an organism to distinguish between distinct environmental events. Behavioral studies of multisensory processing have observed that gains in performance or effects that arise out of multisensory integration are indeed dependent upon the relationship of the component stimuli. Additionally, evidence to support that enhanced sensory integration is correlated with increasing temporal coincidence can be derived from electrophysiological recordings. Meredith et al., 1987 recorded single unit responses in the superior colliculus to temporally varied, paired multisensory stimuli. It was observed that the
largest multisensory gains occurred when the peak neural discharge periods of the unisensory responses overlapped and that increasing the degree of temporal asynchrony between the paired stimuli led to less overlap of the neural discharge periods and less multisensory gain until the point at which no multisensory gains are observed and with further increase in temporal disparity could produce response depression. In order for organisms to perceive the temporal relationships between stimuli, the brain is faced with the challenge of accounting for the differences in both external stimulus and neural propagation that exist for each sensory modality. For example, sound propagates at rate of approximately 340 m/s at sea level, while light propagates at nearly 300,000,000 m/s and somatosensory stimuli require no latent period at all. Upon reaching the organism, the neural processing time for visual stimuli, approximately 50 ms, is actually slower than the neural processing time for auditory stimuli, approximately 10 ms, while the neural processing time for somatosensory stimuli is dependent on neural conduction properties and the physical distance from periphery to the brain. Despite these differences in stimulus energy and neural conductance, perceptual representations emerge that accurately reflect an organism’s environment and allow for the development of temporally coherent multisensory percepts that account for these differences.

**Constructing a Temporal Binding Window**

The concept of a window of temporal integration, during which multisensory interactions occur and coherent multisensory objects are perceived, suggests that intersensory coherence is maintained when stimuli occur in close enough temporal proximity (see Fig. 1A & 1B). The probabilistic epoch during which stimuli are likely to be perceived as simultaneous and thus perceptually bound has been termed the temporal binding window (TBW). Rather than being a single value, the TBW represents a range of time within which stimuli are likely to be perceptually bound. From an ethological perspective, such a flexible specification makes sense as multisensory events happen at different distances, with corresponding differences in visual and auditory arrival times. Moreover, a dynamic temporal range may allow for the ability to interpret and construe meaning from more complex multisensory stimuli, like speech, that exhibit a high degree of variability. Stimuli whose temporal proximity exceeds the duration of the TBW are identifiable as distinct events; however, if the TBW is too large, as has been observed to be associated with some neuropsychopathological conditions, stimuli will be integrated and bound together that should not be associated. The utility of the TBW as a proxy for measuring multisensory integration is demonstrated by the temporal dependence of illusory effects, which occur as a result of multisensory integration. For example, in studies by Shams et al., the sound-induced flash illusion (in which the presentation of two or more auditory beeps with a single flash results in individuals reporting multiple visual flashes), was reported to occur only when the auditory beeps are over a limited temporal window (±100 ms) relative to the onset of the flash.

The temporal binding window may be experimentally measured by a number of behavioral tasks. For the purposes of this review, I will describe the simplest of these tasks, a simultaneity judgment (SJ), from which a TBW for a participant can be experimentally derived (see Fig. 1C). An SJ task is a paradigm that asks participants to respond if stimuli were synchronous or asynchronous. For example, “Did the sound and flash of light occur at the same time or at different times?” For a SJ task, a Gaussian-like curve is fitted to a participant’s report of synchrony at different stimulus onset asynchronies (SOAs). A TBW is obtained from these perceptual reports by measuring the width of the fitted curve or range of SOAs above which report of synchrony was measured to exceed some criteria, for example 75%. Unfortunately, no universal criterion exists for measuring the TBW. The point of subjective simultaneity (PSS) is the peak of the curve that defines the SOA at which a subject perceived a stimulus pair to be maximally synchronous.

**Figure 1. Temporal Integration. A = auditory, V = visual.**

The temporal binding window is a construct that is highly dependent upon the features of a stimulus and dependent on the task used to measure the TBW. One comprehensive study of the TBW parametrically varied stimulus complexity, task criteria, and statistical criteria. This study observed that the TBW is asymmetrical for low-level and
non-speech stimuli, with individuals less likely to report stimuli as synchronous when the auditory stimulus preceded the visual stimulus. In contrast, the TBW for speech stimuli was symmetrical. Reports of an asymmetrical TBW for low-level stimuli likely reflect the relationship of auditory and visual stimulus energies, as visual information always precedes auditory information at their respective sensory modalities. In contrast, a more symmetrical and wider TBW for speech stimuli may reflect additional cortical processing for this specific audiovisual pairing as well as the statistical variability of audiovisual speech\(^5\). Additionally, Stevenson & Wallace also observed that a temporal order judgment (TOJ) task produced narrower estimates of the TBW than SJ or perceptual fusion tasks\(^6\). These findings demonstrate that task and stimulus parameters differences should be considered when comparing estimates of the TBW. Spatial congruence\(^7\), rate of stimulus occurrence\(^8\), and developmental age\(^9\) are additional factors that have been observed to influence perception of temporal synchrony and estimates of the TBW.

**Plasticity of the TBW**

The window of temporal integration and multisensory interaction remains plastic throughout the course of development from infancy to adulthood and is likely altered by increasing experience with natural statistical multisensory stimulus relationships, cognitive representations of multisensory objects, and changes in the relationships between stimuli\(^12\) 13 34 38 42-45. The development of multisensory sensory temporal coherence and the subsequent ability to distinguish multimodal synchrony from asynchrony has been hypothesized to be a critical step not only in the development of multisensory perception, but also a foundational process upon which higher-order cognitive processes develop\(^46\). Lewkowicz and colleagues recently posited that the ability to detect temporal co-occurrence between multimodal stimuli, particularly audiovisual stimuli, is a fundamental perceptual mechanism upon which the development of multisensory coherence and representations of multisensory objects might be bootstrapped (see Lewkowicz, 2014 for comprehensive review of multisensory development and perceptual narrowing)\(^49\). Indeed, the ability to detect synchrony of multimodal stimuli occurs in early infancy\(^47\) and is followed by the ability to utilize other cues such as facial features\(^48\), affect,\(^49\) and other stimulus properties. Further refinement of multisensory temporal representations continues to occur beyond early development although studies investigating this period of development are more limited\(^33\). The TBW remains broad during childhood in comparison to the TBW for adults presented with low-level stimuli\(^39\). Additionally, narrowing of the left side of the TBW (auditory preceding visual) occurs at an earlier stage in development than the right side of the TBW, which continues to narrow through adolescence into adulthood. This suggests that the more ethologically relevant portion of the TBW is slower to narrow, possibly due to the increased statistical variability of the auditory signal following the visual signal\(^32\). Evidence that the refinement of temporal acuity continues throughout adolescence has also been observed for visual-tactile multisensory objects, although adult-like performance for this pairing appears earlier than for audiovisual objects\(^50\). The plasticity of these perceptual processes likely remains because knowledge of the temporal relationships for novel or changing stimuli must be encoded and represented.

The perception of temporal relationships remains plastic even in adulthood. Early reports of perceptual plasticity described the temporal recalibration that is observed following passive exposure to asynchronous stimuli\(^34\) 44 51 52. In one such study, exposure to asynchronous low-level stimuli (light flashes and tones) was observed to shift the PSS, a widening of the TBW (as indexed by widening of the distribution on an SJ task), and increased perception of the stream/bounce illusion, in which the addition of sound at the point in which the paths of two balls cross creates the illusion of the balls bouncing off of each other\(^45\). The finding that exposure to asynchronous stimuli can alter perception has been extended to more complex stimuli, like speech\(^32\), and that this exposure can transfer to the perception of stimulus pairings different from those during asynchronous exposure\(^53\). More recently, multisensory processing has been demonstrated to be sensitive to perceptual learning paradigms\(^33\) 38 53. While early studies of perceptual learning primarily focused on enhancing perception in a single modality\(^44\), there have been recent efforts to extend perceptual training paradigms to enhance perception of multisensory events\(^55\) 56. Powers et al. demonstrated that a perceptual training paradigm, in which visual feedback was provided following participants’ responses on SJ tasks, was capable of narrowing the TBW for low-level stimuli (visual flashes and tones). Furthermore, this study also observed that these training induced changes in temporal acuity were stable one week following the last training session, which suggests that these changes were not merely limited to the days on which training sessions occurred\(^58\). The capacity for perceptual learning paradigms to enhance multisensory temporal acuity and the observed plasticity of the TBW suggests that these paradigms might be capable of enhancing multisensory processing in those with conditions such as autism and dyslexia in which multisensory temporal processing is atypical\(^57\).

**Temporal Influences on the Perception of Audiovisual Speech**
Recently, there has been a growing effort to understand the relationships between multisensory function and higher order cognitive processes, including speech and language. A classic report by Sumby & Pollack provides a compelling illustration of the effect of multisensory integration on audiovisual speech perception (visual speech enhances intelligibility in noisy environment). Like all multisensory processes, the influence of multimodal speech on perception depends upon the temporal relationships between the modalities. One example is the observation that the perception of illusory McGurk precepts is temporally dependent. The McGurk effect, in which auditory phonemes /pa/ and /ba/ paired with conflicting visual phoneme /ka/ and /ga/ respectively are often perceived as the novel fusion phoneme precepts /ta/ and /da/, A study by van Wassenhove et al. reported that fusion resulting in the report of the illusory precept occurred from 30 ms (auditory leading visual) to 170 ms (visual leading auditory), a finding similar to earlier accounts. Additionally, this study further demonstrated using a SJ task that the McGurk percepts were perceived to be synchronous over a similar temporal range to range in which fusion response was reported and the TBWs for the illusory percepts were narrower than congruent presentations of auditory and visual /da/ and /ta/. The differences in the TBWs observed for individual phonemes, including those resulting from perceptual fusion, illustrates that the TBW is determined by multiple factors and likely influenced by both top-down and bottom-up processes in comparison to novel or low-level stimuli, which likely are primarily influenced by bottom-up processes.

As previously described, the ability to detect audiovisual synchrony was posited to be a foundational, early process in development that allows for other cues to influence perception through cognitive processes and contribute to the overall perception of audiovisual speech. Such processes, like the ability to extract phonemic cues (i.e. categorical phonemic visual correspondence with auditory signal), continues to develop into adulthood well past the initial ability to utilize audiovisual synchrony to identify speech. How these various cues influence the temporal integration of audiovisual speech, however, was only recently been examined. The TBW for audiovisual speech has been known since a study by Dixon & Spitz, in which the TBW was larger for speech than non-speech dynamic stimuli. Subsequent studies observed that the TBW is larger for speech stimuli than for non-speech or static stimuli. Although a wider TBW for speech stimuli may be due to the increased variability of speech and the delay of the auditory component occurring with a delay of 100 to 300 ms following the onset of the visual component of speech, the size of the TBW for speech stimuli still appears to be specific to the stimulus.

While the TBW for speech stimuli might experience some degree of narrowing during development, it remains possible that the size of the TBW is maintained by experience. Larger values for the PSS (where visual leads auditory speech) has been observed for participants’ native language compared to a language with little experience, although increasing experience with a non-native language results in a PSS equivalent to the native language. A recent study by Ten Oever et al. observed that congruent or matched audiovisual phonemes have large TBWs than incongruent audiovisual phoneme pairs. This finding suggests that there are top-down influences utilizing the semantic context of disparate inputs to suggest whether the stimuli should be integrated as well as bottom-up influences that reflect more general stimulus features. Perceptual fusion of low-level or novel multisensory stimuli might be influenced by bottom-up processing of the spatiotemporal relationship of the stimuli. The fusion of learned multisensory stimulus pairings influenced by top-down processing that further shape the TBW may provide an advantage in the context of speech perception that would allow for enhanced comprehension of learned phoneme relationships despite variability in auditory or visual signals.

**Deficits of Temporal Multisensory Processing: Implications for Autism**

Autism spectrum disorder (ASD) is characterized by persistent deficits in social communication and social interaction, as well as restricted, repetitive patterns of behavior or interests. Although people with ASD show enhanced abilities on some unisensory measures, most studies observe deficits for tasks requiring integration across senses. From a multisensory perspective, communication impairment in ASD may arise from increased difficulty in perceiving the temporal relationships between auditory and visual stimuli. Such impairment may prevent audiovisual synchrony from being a reliable cue for determining if two stimuli derive from the same event, an association hypothesized to be important for the development of higher order cognitive processes like speech perception. A recent study by Stevenson et al. found that larger TBWs for speech stimuli in children with ASD were correlated with a decreased ability to integrate auditory and visual speech signals to create a perceptual report of the McGurk effect. The finding that larger TBWs, even with low-level stimuli, were correlated with deficits of multisensory speech perception lends both support for this hypothesis and suggests that further study of the temporal relationships of audiovisual speech perception in both typical development and ASD is necessary.
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This study demonstrates that TBW for illusory speech percepts...
resulting from multisensory integration differs from the TBW for the natural presentations of the same speech phonemes. This emphasizes the multisensory influence of speech perception.


This study was the first to demonstrate that a perceptual learning paradigm was capable of narrowing the TBW for multisensory stimuli. Additionally this paper provided insight into the plasticity of the TBW.


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Food for thought: A protective role for vitamin C in Alzheimer’s disease

Shilpy Dixit

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia, affecting almost 6 million individuals in the United States. Though the progression of the disease has been elucidated, the etiology remains unclear. As research in the field has progressed, a number of contributing factors have been identified, including an increase in oxidative damage from age-related oxidative stress1. Age-related cellular dysfunction, environmental exposure and injury causes oxidative stress. As a result, there is a growing interest in the “oxidative stress hypothesis” in AD, in which oxidative damage to lipids, proteins, and DNA is thought to be the driving force behind AD pathogenesis. This hypothesis is supported by observations of increased oxidative damage to these molecules in AD patients1 that precede the classical histopathological features2. Vitamin C (VitC) is a powerful antioxidant that protects against the oxidative damage associated with AD. Studies show the activity of endogenous antioxidant enzymes and levels of nutrient-based antioxidants are significantly reduced in early and late stages of AD3. While little can be done to increase levels of endogenous antioxidants in AD patients, proper diet and supplementation can easily and efficiently increase VitC concentration in the body. The following review will address the ways oxidative stress contributes to AD pathogenesis and the ways in which VitC may be protective.

Keywords oxidative stress, neurodegeneration, Alzheimer’s disease

Competing hypotheses in Alzheimer’s disease pathology

Alzheimer’s disease was first characterized by the presence of amyloid plaques and neurofibrillary tangles (NFTs) by Alois Alzheimer in 1906. These histopathological hallmarks, and mild cognitive impairment (MCI) in most cases, persist today as the criteria for diagnosis of the disease. Amyloid beta (Aβ) is produced by cleavage of the transmembrane amyloid precursor protein (APP) by secretase enzymes. Under normal conditions, APP is cleaved by α- and γ-secretase; however, a shift to the amyloidogenic cleavage of APP by β-γ-secretase has been associated with oxidative stress. The resulting 38-43 amino acid Aβ peptides form insoluble oligomers, which are toxic to cells. The hyperphosphorylation tau inhibits the ability of the tau proteins to stabilize microtubule assembly and promotes NFTs, disrupting intracellular transport. The canonical “amyloid cascade hypothesis” posits that the accumulation of Aβ oligomers generates oxidative stress, which induces tau-mediated neuronal degeneration.

According to the “mitochondrial cascade hypothesis,” age-related and genetic mitochondrial dysfunction results in deficient energy metabolism and an increase in oxidative stress capable of inducing AD pathologies. Recent studies show that significant oxidative damage precedes the classical histopathological features of AD. Further, the accumulation of Ab then contributes to oxidative stress, evidenced by an increase in oxidatively damaged molecules before the formation of Aβ plaques. The marriage of these hypotheses establishes the cyclical relationship between oxidative stress and AD pathologies into the “oxidative stress hypothesis” of AD (summarized in Figure 1).

Oxidative stress and Alzheimer’s disease

The brain is particularly vulnerable to oxidative damage because of its enriched lipid composition and significant oxygen consumption. Oxidants can damage all cellular components, but increased damage to lipids and proteins and DNA is closely associated with AD pathogenesis. Lipid peroxidation refers to the degradation of lipids following oxidative attack. Clinical studies indicate lipid peroxidation is elevated as a result of age, with even greater increases observed in AD brains compared with age-matched controls. Lipid peroxidation end-products, such as isoprostanes, malondialdehyde (MDA), and 4-hydroxynonenal (4-HNE) are highly reactive and cause significant alterations to downstream mechanisms. Several studies show that 4-HNE is able to bind to and inhibit membrane components such as glucose transporters, glutamate transporters and Na/K ATP-ase. Aβ-induced cell death also appears to be mediated through 4-HNE, which has been shown to activate the caspase cascade and mitochondria-mediated pathways.

Oxidative attack on proteins results in structural alterations, loss of function and resistance to degradation. Redox proteomics studies in AD brains have identified...
oxidatively modified proteins associated with ATP production and proteasome activity\textsuperscript{29}. These findings are consistent with the decreased energy metabolism and accumulation of damaged proteins observed in the brains of AD patients\textsuperscript{1}. Oxidant-induced protein glycation reactions form advanced glycation end products (AGEs), which have been implicated in the Aβ and NFT aggregation found in AD\textsuperscript{18,30}. Several immunohistochemical studies have shown AGEs associated with Aβ-plaques and NFT in the brains of AD patients\textsuperscript{31}. Li et al. reported hippocampal neurons treated with Aβ-AGE showed decreased viability, increased tau hyperphosphorylation and apoptosis compared to neurons treated with Aβ alone\textsuperscript{32}. Ab peptides are produced and degraded under normal conditions throughout life; however, increasing evidence has implicated Aβ oligomers in the neurotoxicity associated with AD\textsuperscript{9,33}. Thus, the propensity for protein-protein cross-linking by AGEs support a substantial role for oxidant-induced AGE production in AD pathogenesis.

Alterations in gene expression occur with age\textsuperscript{34}. Even greater alterations to genes involved in oxidative stress, amyloid beta production and inflammation are observed in AD patients compared with controls\textsuperscript{35}. Mitochondrial and nuclear DNA bases are susceptible to oxidative attack, resulting in damage that can alter gene expression\textsuperscript{19}. Studies show that oxidative stress increases β-secretase activity by upregulating the expression of BACE1, the major component of the β-secretase enzyme\textsuperscript{36,37}. AGEs, discussed previously, induce BACE1 expression by activation of the receptor for advanced glycation end products (RAGE). Consistent with evidence that chronic neuroinflammation is observed in AD, RAGE activation induces the inflammatory response in cells\textsuperscript{59}. Epigenetic studies have identified differences in methylation patterns, indicating that this form of gene regulation may contribute to AD\textsuperscript{10}. Maestroeni et al. report hypomethylation in AD between monozygotic twins\textsuperscript{41}, as well as in AD patients compared with age-matched controls\textsuperscript{42}. Similarly, a study by Chen et al. reported that cerebral epithelial cells treated with Aβ(1-40) peptide caused global hypomethylation\textsuperscript{43}. Genes related to AD that are upregulated by hypomethylation and oxidative stress including BACE1 and PS1 (amyloid beta production), as well as TNFα, IL-6 and IL-8 (inflammation)\textsuperscript{40}. Paradoxically, the promoter region of neprilysin, an important protein in amyloid beta degradation, is shown to be hypermethylated in AD patients resulting in decreased expression\textsuperscript{43}, suggesting gene-specific regulation.

**Vitamin C is an essential molecule in the brain**

The activity of endogenous antioxidant enzymes decreases in Alzheimer’s patients, suggesting that the antioxidant barrier is being overwhelmed by increasing oxidative stress\textsuperscript{44}. For this reason, maintaining high levels of nutrient-based antioxidants as a second line of defense may be an essential therapy. VitC, also known as ascorbate, is a vital molecule for its antioxidant and non-antioxidant capabilities. VitC has a low reduction potential, which al-
Vitamin C attenuates oxidative stress

Vitamin C deficiency is surprisingly common, with low-income and the elderly individuals showing the greatest risk\(^\text{51}\). For this reason, the relationship between Vitamin C deficiency and oxidative damage is of particular interest. The *gulo*\(^{-/-}\) mouse model, developed by \(\text{Maeda et al. (2000)}\), possesses an inactive form of the gene for L-gulono-\(\gamma\)-lactone oxidase, the enzyme crucial for the final step of endogenous Vitamin C synthesis in the liver. Like humans, these mice are dependent on dietary Vitamin C, and eventually die after developing scurvy\(^\text{52,53}\). Harrison et al. first established elevated oxidative stress and sensorimotor deficits in *gulo*\(^{-/-}\) mice from *gulo*\(^{+/-}\) dams even though the mice were maintained on sufficient supplementation to maintain physiological Vitamin C levels after weaning\(^\text{53}\). In a follow-up study conducted by Harrison et al., oxidative stress in *gulo*\(^{-/-}\) pups from *gulo*\(^{+/-}\) dams was assessed intermittently from embryonic day 20 (E20) to post-natal day 18 (P18) without supplementation\(^\text{44}\). Vitamin C was significantly lower in the cortex and liver of *gulo*\(^{-/-}\) pups compared with *gulo*\(^{+/-}\) and *gulo*\(^{+/-}\) littermates, and fell precipitously in the cortex of *gulo*\(^{-/-}\) pups from P1 to P18. Interestingly, no significant difference in MDA levels was observed in the cortex of between groups, while MDA levels continued to increase in the liver of *gulo*\(^{-/-}\) pups compared with *gulo*\(^{+/-}\) and *gulo*\(^{+/-}\) littermates. Vitamin C levels in the cortex of *gulo*\(^{-/-}\) decreased rapidly between P1 (5.5\(\mu\)mol/g) and P18 (1.5\(\mu\)mol/g), indicating significant Vitamin C activity in the brain. Liver concentrations of Vitamin C in *gulo*\(^{-/-}\) were consistently much lower from P1 (0.5\(\mu\)mol/g) to P18 (0.1\(\mu\)mol/g)\(^\text{45}\), allowing for significant increases in MDA levels in the liver.

One caveat to consider is that genetic manipulation, though a powerful experimental tool, does not account for changes to synergetic mechanisms that occur through evolution. Guinea pigs, like humans, cannot synthesize Vitamin C due to an evolutionary mutation in the gene for L-gulono-\(\gamma\)-lactone oxidase. This model may resemble homeostatic regulation of Vitamin C and deficiency in humans more closely than the *gulo*\(^{-/-}\) mouse model. A recent study by Paidi et al. investigated the effects of pre- and post-natal Vitamin C deficiency and repletion on MDA levels in the brain\(^\text{55}\). In this study, guinea pig dams were provided Vitamin C supplementation to achieve physiological levels or sub-physiological levels in embryos. After birth, pups were maintained at physiological Vitamin C for the control condition or sub-physiological Vitamin C levels for the depleted condition. A cohort of gestationally depleted animals was supplemented with sufficient Vitamin C to achieve physiological levels for the repleted condition. Brain and plasma Vitamin C was significantly lower in animals in the depleted condition compared with the control and repleted conditions. Depleted animals showed significant increases in MDA and DHA, compared with control and repleted animals, consistent with previous studies. Importantly, no significant difference between control and repleted animals was observed on any measure, which contradicts the assertion of irreversible oxidative damage in the initial *gulo*\(^{-/-}\) study\(^\text{53}\), and highlights possible mechanistic differences between models. These data suggest the direct relationship between oxidative stress and Vitamin C deficiency, and lend support to the protective role of Vitamin C in the brain.

Vitamin C attenuates Alzheimer’s disease pathology

The amyloid cascade hypothesis has dominated AD research due to the presence of A\(\beta\) plaques in AD brains and A\(\beta\) is toxic to cells *in vitro*. The synaptic degeneration and
cell death associated with the cognitive decline observed in AD appears to be mediated through oxidatively modified molecules. Martindale et al. reviewed apoptotic pathways mediated by oxidative stress. Among them, activation of p53, NFκB, and stress-activated protein kinases (SAPKs) such as JNK/p38 appears to favor cell death in response to oxidative damage. Activation of p38 has also been shown to phosphorylate tau, consistent with evidence of p38 localization exclusively to NFT in AD patients.

Huang and May showed that pre-treatment with VitC attenuated Aβ-induced apoptosis in culture. In this elegant study, SH-SY5Y cells accumulated VitC added to culture medium to physiological intracellular concentrations. Treatment with increasing concentrations of Aβ for 1 hour resulted in significant decrease in intracellular VitC, suggesting increased Aβ-induced oxidative stress was depleting VitC stores in these cells. Aβ-induced apoptosis was assessed by three methods: externalization of phosphatidylserine, TUNEL stain and caspase-3 activity. Pre-loading cells with VitC prevented apoptosis according to all three methods. Remarkably, this study also found that cells treated with VitC produced less endogenous Aβ than cells without VitC treatment, suggesting the presence of VitC can prevent activation of the amyloidogenic cascade.

Sufficient evidence exists for the oxidative stress hypothesis of AD and the protective role antioxidants may play; therefore, therapeutic administration of VitC to prevent AD pathogenesis is of great interest to the field. Acute administration of VitC has been shown to improve cognitive deficits in AD mouse models without affecting plaque formation. A study by Murakami et al. investigated chronic VitC administration and Aβ pathology in the AβPP transgenic AD mouse model. In this study, mice were treated with ~1000mg of VitC daily through ad libitum access to supplemented drinking water for six months. At twelve months of age, the mice were assessed for differences in oxidative stress and AD pathology, with particular interest in Aβ assembly. As expected, the VitC-treated group showed decreased levels of protein carbonylation and increased glutathione as markers of oxidative stress. Interestingly, VitC-treated mice showed a decrease in total soluble Aβ42 (ELISA), Aβ dimers (ELISA, Western blot) and phosphorylated tau protein (Western blot) compared with vehicle-treated mice. Congruent with studies employing acute VitC administration, chronic VitC supplementation did not alter plaque formation in AβPP mice. The VitC-treated AβPP group performed more like wild-type controls on Y-maze tasks than did the vehicle-treated AβPP group; with no observed improvement on memory tasks. Kook et al. reported similar histopathological results using a gulo−/−;5XFAD transgenic AD mouse model. In this study, higher VitC supplementation (3.3g/L) reduced overall amyloid plaque burden. This finding is inconsistent with the studies discussed above; however, the mice in this study began supplementation much earlier and carry a greater mutation burden for a more severe phenotype. The authors from either study did not venture to characterize a mechanism by which VitC is protective; however the presence of oxidants has been shown to induce Aβ and neurofibrillary tangle assembly. Thus, VitC may prevent AD pathology by scavenging reactive species before they impact Aβ and tau.

VitC efficacy and clinical studies

Clinical studies regarding VitC protection against AD report mixed results. While some studies measure distinct markers of oxidative stress and VitC, most studies attempt to correlate VitC intake from self-report with cognitive impairment in the elderly and in individuals at risk for AD. Genetic predisposition to AD is attributed to a handful of genes that account for a small percentage of cases. Polymorphisms have been identified in genes involved in mechanisms commonly disrupted in AD that may confer risk. SVCT polymorphisms have been shown to alter VitC concentrations in biological fluids. As discussed previously, neurons contain the highest concentration of VitC in the body due to a number of recycling mechanisms; therefore, biological fluid may not accurately reflect antioxidant activity in the brain. Also, the choices an individual makes over the course of a lifetime may have lasting effects on cognition. The underlying discrepancies arise from the incomparable methods employed by these studies, as well as any number of confounding variables that individualize AD among patients.

Conclusion

The long-held belief in an etiological role of Aβ plaques and NFT in AD is falling out of favor. Alzheimer, himself, believed the histopathological features of the disease were products of some upstream dysfunction rather than the cause. Overwhelming evidence implicates oxidative stress as a major contributor to AD. Cellular mechanisms such as energy metabolism, protein degradation and antioxidant defense become less efficient with age, thus resulting in oxidative stress. As the body of evidence supporting the oxidative stress hypothesis of AD grows, so does research regarding the efficacy of antioxidants in the treatment of AD. VitC is a powerful antioxidant that can directly interact with oxidants, as well as work synergistically with other antioxidant molecules and enzymes. A clear relationship exists between VitC and oxidative stress. As discussed in this review, studies show VitC supplementation protects against oxidative damage and prevents AD pathol-
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Towards an Understanding of Diet Failure: Stress and Feeding in the Extended Amygdala

Tracy Fetterly

Obesity rates have been on the rise for several decades. Unfortunately, being overweight results in an increase in many associated health issues. While weight loss is highly recommended to treat these health risks, long-term weight maintenance has proven to be quite difficult. Although much research has focused on understanding the molecular determinants of body weight, less work has focused on this issue of diet relapse. Human laboratory studies interested in control of food intake have remained largely anecdotal, but suggest a connection between stress and food consumption. Recently, similarities between the brains of addicted individuals and obese individuals have led researchers to investigate the overlap between motivation for drug-seeking and motivation for food seeking, including similarities between relapse to drug-use and diet failure. The drug-seeking reinstatement paradigm has been a useful model for investigating the reinstatement of palatable food-seeking. This review will discuss the drug-relapse model and how it has been converted to studies of palatable food-seeking, particularly focusing on stress-induced reinstatement. Additionally, the role of the extended amygdala, a region implicated in stress-induced reinstatement of drug-seeking, will be examined. The discussion will focus on the role of extended amygdala corticotropin releasing factor (CRF) in controlling feeding behavior and how this system may be altered during caloric restriction, resulting in vulnerabilities to high-fat feeding.

**Keywords** Obesity, diet failure, extended amygdala, bed nucleus of the stria terminalis, corticotropin releasing factor, stress-induced reinstatement

Obesity rates have been increasing for several decades in both the United States and globally. This obesity epidemic poses a significant problem, as increased fat mass is a risk factor for many health risks including cardiovascular disease, metabolic syndrome, and type II diabetes. Weight loss is commonly recommended as a treatment for these health problems. Even losing just 10% of body weight can greatly improve the health status of individuals. While 10% weight loss is not always enough to remove the individual from the obese category, the health benefits are evident. Unfortunately, weight loss has proven difficult to maintain, with studies estimating that only 20% of people are able to maintain significant weight loss (≥10% of body weight) that persists for at least one year.

While studies have begun to address the characteristics of people who successfully keep off weight, few reports have investigated those who are unsuccessful. A study in 2013 asked patients who were about to undergo bariatric surgery to self-report what they considered the reason for weight gain. The two most common variables cited were stress (63%) and dieting (56%). This connection between stress and restricted eating has been studied in laboratory settings looking at human feeding behavior. Earlier studies were interested in the differences in feeding behavior between obese and healthy individuals. One such study used difficult logic problems to depress the moods of individuals before they were given crackers to rate for flavor and appeal. The initial hypothesis was that obese individuals would eat more when mood was depressed. Surprisingly, dieting habits, and not obesity status, were found to be the best predictor of eating responses to a depressed mood. Dieters consistently ate more after mood had been lowered than non-dieters. Other studies have continued to point to dieting habits as a predictor for eating behavior. In one review addressing multiple hypotheses concerning stress-induced eating, the authors noted that restrained eating was the most consistent predictor of vulnerability to stress-induced eating in women.

Interestingly, eating as a response to stress may not be generalized overeating; instead, research has pointed towards overeating of specifically highly palatable foods following stress. When exposed to stressful videos and given access to food in a laboratory setting, women prefer sweet food under the stressed condition. Another study analyzed high and low stress reactors, as determined by cortisol response to stress. The high stress reactors not only ate more food in response to stress, but they specifically consumed...
sweet food⁹. Such studies have led others to hypothesize that stressed conditions could result in an enhanced sensitivity to highly palatable foods and that certain individuals may be more sensitive to the reinforcing effects of such foods¹⁰,¹¹, suggesting an important link with central reward pathways¹².

An interacting of feeding behavior with the reward pathways sets up potential similarities between feeding behavior and drug-seeking behavior. Relapse to poor eating habits following dieting may indeed be similar to reinstatement of drug-seeking behavior, including the role of stress as a trigger. This review will discuss evidence for the shared characteristics between feeding behavior and drug-seeking behavior, focusing on the role of the extended amygdala and corticotropin releasing factor (CRF).

Reinstatement of Drug-Seeking Behavior

Understanding the mechanisms controlling relapse to drug-seeking has been an area of investigation for many years. A rodent model of reinstatement to drug-seeking as a way to model relapse to drug-use has been well defined in the field. Commonly this model involves first training the animals to lever press to receive a dose of drug, then extinguishing this behavior by no longer providing drug in response to the lever, and then finally manipulating the situation to induce reinstatement of the lever pressing (Figure 1). One of the earliest studies using this paradigm showed that re-exposure to the drug administered throughout the training phase would result in reinstatement of the drug-seeking behavior¹³. Researchers then began to investigate what other methods could reinstate the behavior without actually providing the drug. Several types of cues were shown to illicit the desired reinstatement, including exposure to stressors¹⁴-¹⁷.

Drug users often report stress as one of the major factors leading to relapse¹⁸, making the use of stressors in the reinstatement paradigm potentially applicable to human behavior. The definition of stressor can vary throughout the literature, but multiple stressors are capable of inducing reinstatement of drug-seeking behavior. One of the first stressors used was foot-shock to reinstate heroin seeking in rats¹⁴. In addition to foot-shock, the pharmacological stressor yohimbine has been used to induce reinstatement. Yohimbine induced reinstatement of drug-seeking has been shown for multiple substances of abuse including methamphetamine, alcohol, and cocaine¹⁵-¹⁷.

The mechanism of action for stress-induced reinstatement of drug-seeking remains an open question, but some of the important molecules and brain regions have been identified. Corticotropin releasing factor (CRF) is the neuropeptide responsible for initiating the stress axis, also known as the hypothalamic-pituitary-adrenal (HPA) axis. In response to a stressor, CRF is released from the hypothalamus, where it can then act on the pituitary to release adrenocorticotropic hormone, which stimulates the adrenal glands to release corticosterones. One common definition of a stressor is any action that results in the release of corticosterones into the bloodstream. However, CRF is also expressed in other regions of the brain outside of the hypothalamus, and therefore can also mediate actions within the central nervous system.

Based on its role in responding to stressors, CRF has been studied in drug relapse models interested in stress-induced reinstatement of drug-seeking. Intracerebroventricular (ICV) injection of a CRF receptor antagonist, D-Phe, has been shown to block foot-shock induced reinstatement of cocaine-seeking¹⁹. Injection of D-Phe directly into the bed nucleus of the stria terminalis (BNST), part of the extended amygdala, will also block reinstatement. Injection of D-Phe into the central/basolateral amygdala does not alter reinstatement, suggesting a specific role for BNST CRF in stress-induced reinstatement. Similarly, CRF injections into the
BNST can also induce cocaine-seeking without the presence of an outside stressor\textsuperscript{19}. These results have been supported by the use of another CRF receptor antagonist, CP-154,526. Injection of CP-154,526 into the BNST, but not the amygdala or nucleus accumbens core can attenuate foot-shock induced reinstatement of morphine conditioned place preference\textsuperscript{20}. This once again demonstrates the specific effects of BNST CRF. Evaluating CRF mRNA levels before and after exposure to stressors provides further evidence for the role of BNST CRF in stress-induced reinstatement. Both foot-shock and yohimbine were shown to increase CRF mRNA levels specifically in the BNST\textsuperscript{21}. Furthermore, the CRF receptor antagonist antalarmin has been shown to block yohimbine-induced reinstatement of alcohol seeking\textsuperscript{22}. Antalarmin does not alter corticosterone release, suggesting the role for antalarmin in blocking reinstatement is independent of the HPA axis.

These studies point towards the importance of extra-hypothalamic CRF, and particularly the extended amygdala, in the stress-induced reinstatement model of drug relapse. As discussed above, CRF remains important for multiple drug classes, multiple stressors, and multiple experimental paradigms\textsuperscript{19-23}. The behavioral and pharmacological evidence for the role of extended amygdala CRF in drug-seeking has been further supported by electrophysiological work\textsuperscript{24-28}. For instance, CRF in the BNST can regulate BNST neurons that project to the ventral tegmental area (VTA), a region involved in the reward pathway\textsuperscript{25}. Additionally, these circuits are modulated by ethanol exposure and withdrawal. The evidence for a role of BNST CRF in drug relapse has remained consistent throughout many studies utilizing a wide array of research techniques.

The Overlap Between Addiction and Obesity

One hallmark of addiction is the continued use of substances despite adverse consequences. For example, smokers are aware of the health risks associated with cigarettes, but still continue smoking. Similarly, obese individuals understand the health risks connected to being overweight, but the overconsumption of food persists. Volkow and Wise point out that just as not all people exposed to drugs become addicted, not all people that eat high-fat food become obese\textsuperscript{29}. Brain imaging studies that assess dopamine receptor (D2) availability have shown similar reductions in striatal D2 receptors in obese subjects and addicted subjects. Additionally, there is evidence for food addiction with studies showing rodent models of sugar dependence\textsuperscript{30}. The similarities between feeding and addiction continue into the realm of relapse. Researchers have begun to use the reinstatement model of drug relapse described above to look at feeding behavior, with potential implications for better understanding diet relapse.

Reinstatement of Food-Seeking

The drug-reinstatement paradigm has been successfully converted for use in assessing food-seeking reinstatement. Rodents are trained to lever press for food pellets. This behavior can be extinguished and then reinstated using multiple methods of reinstatement (Figure 1). Differing results have been obtained when using different types of food rewards and various states of caloric restriction. Reinstatement has been shown to be much stronger when using more palatable food (pellets that contain some amount of fat)\textsuperscript{30-32}. When food pellets are completely devoid of fat content, reinstatement of food-seeking has been less successful\textsuperscript{31}. Sucrose has also been used in this paradigm, but differences between the use of sucrose and the use of food pellets have been observed. This includes differential responses to reinstatement methods\textsuperscript{32} and pharmacological manipulations\textsuperscript{32-34}. This review focuses on the use of the high-fat diet because this parallels the high fat content found in the Western diet consumed by humans. Additionally, manipulating caloric intake before the paradigm can also result in differing levels of reinstatement. The most consistent results have been observed when using rats that are calorically restricted\textsuperscript{35}. Unlimited access to chow has been allowed in some experiments, but these experiments tend to be harder to replicate\textsuperscript{32,33,35}.

These methodological differences may further support the comparison of drug-use relapse to diet relapse. Just as saline injections during the training phase will not result in reinstatement later, fat-free chow will also not lead to reinstatement. This could be due to differences in the reward value between fat-free and high-fat food. Additionally, the human evidence, as discussed in the introduction, for stress-induced eating of highly palatable foods supports the use of high-fat food in the reinstatement paradigm. The increased response of calorically restricted rodents to the paradigm also matches the human studies showing a vulnerability of restricted eaters or dieters to stress-induced feeding.

Multiple methods of reinstatement have been utilized in the food-seeking paradigm. These methods are based off known ways to induce drug-seeking reinstatement and include food priming, cue priming, and stress. Yohimbine, the same pharmacological stressor utilized in drug relapse literature, can indeed reinstate palatable food seeking in the rat relapse model\textsuperscript{30}. CRF has also been implicated in this relapse to food-seeking. The CRF receptor antagonist antalarmin can block yohimbine-induced food-seeking reinstatement\textsuperscript{36}. As discussed above, the drug reinstatement literature has already provided evidence that antalarmin acts independently of the hypothalamic actions of CRF\textsuperscript{22}. Therefore, extrahypothalamic
CRF may also be playing a role in food-seeking behavior. The extended amygdala, a region of high CRF expression, is one area implicated in the control of feeding behavior.

**CRF and Feeding in the Extended Amygdala**

The connection between CRF and feeding first began with observations of stress-induced hypophagia. Multiple stressors have been shown to induce hypophagia including restraint stress and ICV injection of CRF. The role of CRF signaling was further confirmed when alpha-helical CRF, a competitive antagonist of CRF, was shown to partially reverse hypophagia induced by both CRF injection and restraint stress. CRF induced hypophagia can also be reversed by ICV injection of an endogenous ligand of the opioid N/OFQ receptor, which is known to have anti-CRF actions. Additionally, injection of this ligand directly into the BNST will reverse the feeding decrement, while injection into the central amygdala, locus coeruleus, ventral medial hypothalamus, paraventricular nucleus, and dorsal raphe had no effect. The BNST, which receives a large noradrenergic input, also plays a role in β-adrenoreceptor mediated decreases in food intake, providing further evidence of a link between the BNST and hypophagia.

Despite the clear anorectic role of CRF signaling in the BNST, CRF may also be playing a role in inducing palatable food seeking in certain circumstances. In one experiment, rats were given both regular chow and the highly palatable high-fat chow. The rats were then chronically stressed using repeated bouts of restraint stress. Overall, the stressed rats showed a decrease in feeding confirming the work discussed above. However, the stressed rats increased the percentage of high-fat food that was consumed as compared to control groups. Additionally, control rats that had access to the high-fat diet had decreased CRF mRNA levels when compared to rats eating only the regular chow. In a follow-up study, CRF mRNA levels were reported to decrease specifically in the hypothalamus and the BNST following palatable food consumption. They propose that consuming high fat food can result in a lessening of the feeling of stressors via this decrease in CRF mRNA. These studies suggest that the role of CRF in feeding is not straightforward, but is instead dependent on previous experience.

If previous experience is able to shape the role of CRF in feeding, then it is important to understand how overeating and dieting are altering the CRF system. A study published in 2010 addressed the effects of caloric restriction on the CRF system. This study calorically restricted mice, resulting in 10-15% weight loss, which mimics human diets. Following this restriction, a significant decrease in CRF mRNA was observed specifically in the BNST. This change in mRNA levels persisted even after one week of re-feeding in which mice returned to their original weight. Additionally, the previously restricted mice were prone to stress-induced eating of palatable food. Stressing previously restricted mice resulted in a significant increase in binge eating of highly palatable food. This suggests that caloric restriction, or dieting, could be altering sensitivity to stress.

On the other side of the spectrum, overconsumption can also affect the stress system. One study placed mice on a high-fat diet for six weeks. Following the high-fat diet, mice exhibited a hypersensitivity to stress. Additionally, when the mice underwent high-fat diet withdrawal, such as may occur during dieting, the mice showed enhanced motivation for both sucrose and high-fat food rewards. From these studies, it is clear that cycles of dieting and overeating are indeed resulting in alterations in the stress system, including the CRF system in the extended amygdala. These changes may in fact be increasing sensitivity to stress-induced eating.

**Conclusion**

While much research has focused on better understanding what drives eating and how body weight is determined, relatively little work has been done to understand how this system is altered during cycles of overeating and caloric restriction. Better understanding of these changes will be important in the future for finding ways to improve the duration of weight loss.

One big challenge in the field is modeling the failed diet. Observing the overlap between the addicted individual and the obese individual has allowed feeding researchers to adapt techniques long used in the highly studied addiction field. The use of the drug relapse reinstatement model as a way to model palatable food-seeking has helped researchers to better characterize vulnerabilities to high-fat feeding. This paradigm allows one to investigate the motivation of mice for highly palatable food under various states of caloric restriction. Additionally, this model has shown the different types of stimuli that can induce reinstatement of palatable food-seeking, providing support for the concept of stress-induced eating.

Just as in drug reinstatement, the actions of CRF are seemingly important for the control of feeding. In particular, extrahypothalamic CRF has been shown to play a role in food-seeking reinstatement. Further experiments addressing the role of CRF in feeding have shown its anorectic actions...
and that a region of the extended amygdala, the BNST, may mediate these actions. However, the CRF system appears to play a more dynamic role than simply inducing decreases in feeding. Instead, this system can be altered by various states of feeding including caloric restriction and high-fat feeding. These changes may actually result in an increase in the drive for high-fat food reward and an increase in sensitivity to stress-induced eating.

The studies discussed above were largely behavioral studies that utilized pharmacology to better understand which molecules may be important. While it is clear that the CRF system in the extended amygdala is important for understanding sensitivities following high-fat feeding and/or caloric restriction, very little is currently understood about the circuitry of this system. In the future, it will be important to dissect the circuits controlling the extended amygdala CRF system. An understanding of this circuitry will make it easier to target this system in an attempt to increase the success rates of long-term weight loss.

References


This paper is from our lab and utilizes some of the same electrophysiological techniques and the same reporter mouse line that I will be using for my research. Additionally, the paper provides electrophysiological evidence of how CRF can modulate BNST neurons that project to reward circuitry.


This paper shows that the pharmacological stressor yohimbine can restate food-seeking behavior, confirming similarities between reinstatement of drug-seeking and food-seeking. Additionally, this paper shows reinstatement can be blocked by a CRF receptor antagonist, providing a link between the CRF system and food-seeking behavior.


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This paper shows that caloric restriction alters CRF mRNA levels in the BNST. Additionally it shows that previous caloric restriction can increase binge eating following a stressor. This sets up the potential that dieting could be altering the CRF system in the BNST, making individuals more prone to palatable feeding.


**Further Information**

http://www.mc.vanderbilt.edu/root/vumc.php?site=winder
Stress can be Hazardous to Your Health: ER Stress in Charcot-Marie-Tooth Disease

Rose Follis

Charcot-Marie-Tooth (CMT) disease, or hereditary motor and sensory neuropathy (HMSN), is a group of inherited peripheral neuropathies which, collectively, affect 1 in 2500 people in the United States. CMT is characterized by progressive axonal degeneration, often coupled with myelin degradation and Schwann cell dedifferentiation, in distal limbs. As a consequence, patients suffering from CMT exhibit progressive tactile sensory loss and motor deficits. Despite 160 years of research, there is no approved treatment for CMT, making development of targeted drug therapies a priority. Recent observations suggest signaling in the Endoplasmic Reticulum (ER) contributes to CMT symptomology in some variants of the disease. The accumulation of mutant proteins in the ER of effected Schwann cells triggers the activation of the Unfolded Protein Response (UPR), a series of signaling cascades that adapt the cell to stress, and return the ER to homeostasis. However, prolonged UPR activation is associated maladaptive responses, and in Schwann cells appears to contribute to CMT pathology. In fact, the relief, or reduction, of UPR pathway activation has been associated with an alleviation of CMT-like motor deficiencies in three models of the disease. Unfortunately, very little is known about the mechanism/s by which ER stress, and the UPR, contribute to CMT pathology. Ultimately, a better understanding of ER stress mechanisms in Schwann cells must be achieved before comprehensive therapeutic strategies can be developed.

Keywords: Endoplasmic reticulum stress, charcot-marie-tooth disease, schwann cell, unfolded protein response, myelin

Charcot-Marie-Tooth (CMT) disease, or hereditary motor and sensory neuropathy (HMSN), is the most common inherited neuropathy, with an estimated prevalence of around 1 in 2500 individuals. First characterized in 1886 by French neurologists Jean-Marin Charcot and Pierre Marie, with a nearly simultaneous independent report by Howard Tooth, CMT is characterized by progressive axonal degradation of the peripheral motor and sensory nerves. As a result, clinical symptoms include sensory loss and muscle weakness with eventual atrophy in distal limbs. Unfortunately, despite several proposed therapies, no effective, targeted treatment for CMT is available. Therefore, disease management is limited to physiotherapy, corrective surgery, and for the 20-30 percent of patients who suffer from neuropathic pain, analgesics.

However, due to the rapid advancement of clinical genetic testing and small-scale imaging, there is significant progress towards determining the causative mutations and basic pathology of the disease. As a consequence, CMT is now recognized as a heterogeneous group of, inherited neuropathies, and a sub-classification system is utilized based on site of mutation, cell type of origin, and inheritance pattern. One of the first discoveries, based on neurophysiological studies, was that there are two categories of CMT patients. The vast majority (classified under CMT1,3, and 4) display severe reductions in motor conduction velocity(MCV) of the ulnar nerve, with a MCV of less than 38m/s. For comparison, the typical MCV in the ulnar nerve is 50-60m/s. Patients with CMT2 show less abridgment of MCV and have CMT prompted by axonal mal-function. Through nerve biopsy and Schwann cell grafting experiments, patients with the more severe phenotype were later determined to be suffering from a primarily demyelinating disorder, originating from malfunction in Schwann cells.

During development, Schwann cell precur-sors (SCPs), responding to signals from peripheral axons, undergo a highly coordinated maturation process. This results in the formation of a multilayer myelin sheath. The sheath, comprised of a modified and compacted dual lipid membrane, is established around all motor and sensory axons over 1 micron in diameter. Each axon has multiple concurrent myelinating Schwann cells associated with it, allowing for the formation of specialized areas, termed nodes of Ranvier, between them. These areas are rich in ion channels and support the propagation of electric charge along the axon, which coupled with the resistance provided by the compact myelin, forms the basis of saltatory conduc-
In the Schwann cell, the production of myelin relies on the tightly regulated but expansive synthesis of specialized glycosylated myelin proteins and general membrane lipids to generate and maintain the myelin sheath. In fact, it is estimated that Schwann cells produce around 2000 times more cubic millimeters of membrane than an average cuboidal epithelial cell.

Interestingly, many of the causative mutations in the demyelinating form of CMT are pinpointed to regions corresponding to the regulation and coding region of the specialized myelin membrane proteins found in the compacted myelin structure. Peripheral Myelin Protein 22 (PMP22) is a tetraspanning membrane glycoprotein of unknown function which makes up only 2-5% of compact myelin. However, more than 70 percent of patients with demyelinating CMT are classified as having CMT1A, caused by a 1.5 Mb duplication at the p11.2 locus of Chromosome 17. The CMT1A duplication includes a segment spanning the promoter and gene body of PMP22. An additional 20 percent of patients have CMT1B, a classification reserved for point mutations in Myelin Protein Zero (MPZ/P0). MPZ is a membrane glycoprotein of the immunoglobulin superfamily and makes up 50 percent of compact myelin. More than 100 different MPZ point mutations are known to cause CMT1B. Additionally, around 80 other genes are implicated in less common forms of CMT. However, it is estimated that 20-30 more causative CMT genes are yet to be identified.

Although many of the relevant genes in CMT are characterized, much less is known about the progression of aberrant demyelination in CMT. Nerve biopsies from patients reveal that structural anomalies are often present before the onset of symptoms, most frequently abnormally thin myelin, or hypomyelination. This suggests that myelin integrity is reduced during or soon after development. Additionally, one of the key phenotypes of CMT1A (and some cases of CMT1B) is the presence of distinctive onion bulb formations, where several additional layers of loosely organized membranes appear around an axon. These formations occur when a Schwann cell undergoes repeated, incomplete attempts at remyelination. Generally seen in cases of nerve injury, Schwann cells contain a unique ability to dedifferentiate into an immature state and remyelinate. During differentiation, the production of myelin related proteins is halted, myelin is dismantled and the Schwann cell re-enters the cell cycle, cueing proliferation and the expression of axonal trophic factors. Subsequently, Schwann cells undergo a repeating cycle of myelination. It is this process that rescues nerve function following peripheral nerve injury. However, it is suggested that repeated remyelination attempts undergone in CMT cases compromise the effectiveness of the myelin sheath and significantly contribute to axonal degradation.

The signaling pathways responsible for initiating the aberrant dedifferentiation of Schwann cells remain elusive, but one theory with substantial supporting evidence implicates signaling in the Endoplasmic Reticulum (ER). PMP22, PMZ, and other membrane components are transferred to the ER lumen in conjunction with transcription. These proteins then undergo folding and post-translational modifications, such as glycosylation, before being transported to the myelin membrane. Under normal conditions, the majority (~80% in myelinated DRG co-cultures) of PMP22 generated is rapidly degraded in the ERAD degradation system. The chromosome 17 duplication present in CMT1A leads to increased production of PMP22, which leads to increased ER retention and the formation of cytosolic aggregates with ER associated chaperone proteins. Multiple MPZ mutations are also implicated in abnormal ER retention. These observations are important as the ER of myelinating cells under normal conditions already produces an abnormal volume of membrane proteins. The abnormal accumulation of mutant额外 proteins is thought to increase the susceptibility of Schwann cells to ER stress and subsequent activation of the Unfolded Protein Response in many diseases of the central and peripheral nervous system.

ER stress is a metabolic state that is evoked when misfolded or unfolded proteins accumulate abnormally in the ER and overwhelm the available folding machinery (see Figure 1). A major component of ER stress adaption is the activation of the Unfolded Protein Response (UPR). The UPR is a series of signaling cascades activated when misfolded or unfolded proteins bind to molecular chaperones such as BiP (immunoglobulin binding protein) in the ER lumen. As BiP binds the unfolded proteins, it separates from the regulatory domains of the UPR canonical sensor proteins; IRE1α (inositol-requiring transmembrane kinase/endonuclease 1), PERK (Eukaryotic translation initiation factor 2-alpha kinase) and ATF6 (activating transcription factor-6). The sensors are then susceptible to activation, with PERK and Ire1α undergoing phosphorylation, and ATF6 translocating to the Golgi apparatus and cleaved by proteases. Once activated, the sensors trigger an extensive range of signaling cascades which decrease protein synthesis, increase chaperone production, and trigger ER expansion, with the ultimate goal of returning the ER to homeostatic conditions. Briefly, stimulation of the PERK pathway leads to the activation of the EIF2α complex (eukaryotic initiation factor 2) and, together with the production of the CHOP (C/EBP homology protein) and ATF4 (activating transcription factor 4) transcription factors. Additionally, EIF2α blocks translation of most of mRNA products, including those involved in lipid biosynthesis. Ire1α has dual roles as a kinase which phosphorylates several other kinases,
among them TRAF2 (TNF receptor-associated factor 2), and as an endonuclease. The RNase domain cleaves the mRNA of the XBP1u (X-Box binding protein 1 unspliced) transcription factor in the cytoplasm, splicing out an additional exon, and causes the production of XBP1s, a potent transcription factor. It also serves to splice regulatory microRNA. The cleavage of AFT6 by S1P/S2P (sphingosine-1/2-phosphate) causes the release of its N-terminal domain into the cytoplasm, itself a transcription factor. The timing of the UPR determines its effects on cell fate. In many systems, once UPR activation is prolonged past the point of homeostatic recovery, accumulation of CHOP pathway components cues apoptotic programing through unknown mechanisms. The first study implicating ER stress in CMT characterized UPR activation in a mouse model of CMT1B. The model is a transgenic for a MPZ mutant with a deletion of serine 63 (P0ser63del) When expressed in tandem with WT MPZ, it mimics a heterozygous mutation found in patients with a ser very early onset form of CMT1B. Using this model, BiP and CHOP are up-regulated in both P0ser63del MPZ heterozygous and homozygous mice. Also, as with many other MPZ mutants, there is abnormal retention of a partially unfolded form of the protein within the ER lumen of affected Schwann cells. Furthermore, the authors found evidence of pathway activation for all three canonical UPR sensors. Interestingly, breeding heterozygous P0Ser63del MPZ mice to CHOP deficient mice (CHOP-/-) ameliorated the severity of motor deficiency. However, while morphological evidence of demyelination, including onion bulb formations were reduced, the mice still displayed signs of extensive hypo-myelination, indicating that the rescue was incomplete. Also, while CHOP is generally associated with apoptotic programing, both patients and mice with P0ser63del MPZ present with low levels of Schwann cell apoptosis, and apoptosis is not reduced in CHOP -/- mice. The results suggest that, rather than prompting a return to homeostatic ER function, some UPR pathway activation appears to contribute to CMT disease symptomology.

To determine what CHOP associated pathway components were involved in the maladaptive response to P0ser63del MPZ, a follow up study was conducted which investigated the molecular mechanism of the rescue. Comparing the transcriptional profiles of sciatic nerve of rescued P0ser63del MPZ/CHOP-/- with P0ser63del MPZ mutants revealed a transcriptional up-regulation of a number of pathways associated with inflammation, protein folding, and the UPR. Importantly, a protein called Gadd34, the catalytic subunit of a complex responsible for EIF2α dephosphorylation, was up-regulated in P0ser63del mice, but down regulated in the CHOP-/- rescue. Further investigation revealed Gadd34 was an effector of the PERK pathway activation. In P0ser63del MPZ mice, Gadd34 levels increased due to a combination of CHOP transcriptional up-regulation and a rise in Gadd34 mRNA translation caused by the EIF2α positive regulation. The increase in Gadd34, in turn, led to a decrease in the amount of activated EIF2α. The negative feedback inhibition prompted the resumption of translation and attenuation of the PERK pathway. Additionally, experiments crossing P0ser63del MPZ mice with a transgenic line carrying a deactivated Gadd34, reproduced and improved on the CHOP rescue. Not only was motor function improved, but there was an increase in myelin thickness to near WT levels. The authors suggest that the premature attenuation of the PERK pathway by Gadd34 prevents ER adaption to the mutant protein accumulation.

Activation of the UPR was also later noted in the R98C, arginine 98 to cytosine, MPZ mutant mouse model of CMT1B, where a knock-in of the mutant R98C MPZ gene was targeted to the endogenous MPZ gene promoter. Like the P0ser62del MPZ mutant, R98C is associated with cases of CMT1B in humans. However, the clinical phenotypes present in humans with the R98C mutation differ from the P0ser63del phenotype. R98C associated CMT is generally noted to present with early onset of symptoms and hypo-myelination, but lacks classical onion bulb formations. Upon examination, investigators found an increase in c-Jun positive...
Schwann cells during peak myelination. C-jun is transcription factor that serves as negative regulator of myelination, indicating that differentiation was interrupted/regressing. Additionally, as with the P0ser63del MPZ mutant, the R98C MPZ mutant is retained in the ER, and the activation of all three canonical UPR branches is evident. However, the elimination of CHOP, through crossing R98C MPZ mice with CHOP−/− mice, does not alleviate either the motor deficiency, nor the morphological abnormalities. Instead, when R98C MPZ mice were treated with orally administered curcumin, a drug derived from the curry spice, turmeric, there was an amelioration of motor symptoms and UPR activation. Curcumin is a molecule with pleotropic effects, activating multiple anti-inflammatory, immune, and anti-oxidant pathways. It is also known to incite the alleviation of ER stress by facilitating the transport of proteins out of the ER, and had been previously shown to reduce PMP22 aggregation in transfected Hela cells. However, the mechanism by which it accomplishes this is still unknown.

Evidence for the role of ER Stress and UPR associated pathways in CMT1A models is more tenuous. One study demonstrated that treatment of the C22 mouse model (a transgenic line with extra copies of PMP22) with a Heat shock protein 90 (HSP90) inhibitor improved myelin formation. The HSP90 inhibitor, EC137, prompts the enrichment of ER heat shock protein (HSP) chaperone levels, which are known to aggregate with superfluous PMP22 in the cytosol of CMT1A effected Schwann cells. At this time, no reports show canonical UPR pathway activation in similar models of CMT1A. However, UPR activation is evident in the Trembler-J (T rJ) model. One of the oldest models of CMT, the T rJ point mutation of PMP22 is a spontaneous leucine 16 to proline mutation, first characterized in mice in 1951. T rJ heterozygous mice present with an early demyelinating CMT1A-like phenotype, including onion bulb formation. Patients with the T rJ mutation exist, but the UPR activation in these cases is still unknown. Okamoto et al. found BiP and CHOP were up-regulated in 4 month old T rJ sciatic nerves, but no activation of PERK and ATF6 was reported. However, the presence of XBP1s was detected, suggesting Ire1α was activated. Also, as with the R98C MPZ mice, treatment of the T rJ mutants with curcumin lead to an improvement in motor performance and myelin thickness.

Although recent evidence suggests that activation of UPR pathways profoundly impact CMT symptomology, the mechanism(s) by which it effects myelin regulation are unknown. One theory is that continuous activation of the UPR prevents the level of lipid and membrane protein biosynthesis required in the Schwann cell to adequately maintain the myelin sheath, over time this could cause hypomyelination, and once the maintenance of myelin is no longer tenable, demyelination is initiated. This is supported by the down regulation of MPZ and other membrane proteins in the R98C MPZ model when UPR activation is present. However, the adaptive response seen in the P0ser63del model suggests that prolonged UPR activation could accommodate the biosynthetic requirements of the cell.

Another theory is that, in some cases, UPR activation cues the aberrant stimulation of pathways instrumental to the regulation of Schwann cell differentiation and myelin formation, triggering recurrent demyelination. Intriguingly, several general cell stress pathways well known to be activated by the UPR in other systems are key regulators of Schwann cell maturation and dedifferentiation. Examples include; nuclear factor-kappa B (NFκB) and the mitogen activated protein (MAP) family Kinases p38, c-jun N-terminal kinase (JNK), and extracellular signaling related kinases 1/2 (ERK1/2). For instance, the presence of ERK1/2 is required for both Schwann cell precursor maturation, and later for the regulation of myelin formation. ERK1/2 activation is also up-regulated during Wallerian degeneration. Similarly, NFκB acts as a positive regulator in myelination and is activated by nerve injury. JNK and p38, however, are known to activate c-Jun, a potent negative regulator of myelin, but are also activated in Wallerian degeneration. Intriguingly, although ERK1/2 are known as positive regulators of myelin, cueing the up-regulation of ERK1/2 prompted mass Schwann cell dedifferentiation in myelinated Schwann cell/neuronal co-cultures. Induction of p38 presents similar effects in culture, demonstrating that aberrant activation of both positive and negative regulators of myelin formation could potentially drive demyelination. However, the network of interactions between UPR pathway and cell stress pathway in Schwann cells has yet to be fully elucidated, so it is not known whether general stress pathways are up-regulated by the UPR in vivo.

Conclusions

Due to the variety of heterogeneous mutations that cause demyelinating CMT, and the variation in clinical and cellular pathology associated with them, it is highly unlikely one set of molecular pathways is responsible for all cases of CMT. Nevertheless, the presence of ER stress and UPR pathway activation, coupled with the positive results of UPR targeted treatment in several models of CMT, suggests that a greater understanding of ER stress in Schwann cells is needed. Especially, since it could be a key component in developing targeted, effective treatments for patients suffering from CMT. Additionally, if UPR activation is leading to demyelination through pathways regulating Schwann cell maturation and
myelination, further research of the UPR could lead to a better understanding of the dedifferentiation process, and enlighten the investigation of other causative molecular pathologies.

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CANDIDATE REVIEWS

GABAergic Motor Neuron Synaptic Remodeling in C. elegans Revisited: A Look from Postsynaptic Acetylcholine Receptors

Siwei He

The study of synaptic plasticity is one of the most active areas of neuroscience research. The past two decades led to significant progress toward understanding the underlying mechanisms of synaptic plasticity and its critical role in learning and memory formation. Postsynaptic plasticity is a complex biological process involving the insertion/removal of neurotransmitter receptors and their associated proteins, as well as the remodeling of cytoskeletal structures. In this review, I will focus on acetylcholine receptors in both mammals and C. elegans, and discuss their biosynthesis, trafficking and related modulation mechanisms with the goal of providing a general background for understanding the postsynaptic remodeling of cholinergic receptor complexes.

Keywords  postsynaptic, plasticity, AChR, GABAergic motor neuron, C. elegans

Introduction

Synapses are highly specified, asymmetric structures composed of the pre-synaptic axon terminal, synaptic cleft and postsynaptic compartment. Information flow from the presynaptic neuron to postsynaptic cells is mediated by specific chemical neurotransmitters. The plastic nature of synapses that modifies the strength of these neuronal interactions is thought to underlie learning and memory formation. After initial assembly, synapses can undergo extensive processes of structural and functional remodeling known as synaptic plasticity. This phenomenon is regulated by multiple factors, including transcriptional control, neuronal activity, and a variety of secreted signals. Examples of synaptic plasticity include Long term potentiation (LTP) and long term depression (LTD) and respectively involve the insertion and removal of AMPA receptors from the dendritic spine in an activity-dependent manner. These studies brought insight into how glutamate receptors are trafficked between the postsynaptic membrane and cytosolic compartment, and how postsynaptic scaffold proteins (i.e. PSD-95) and cytoskeletal proteins (i.e. actin), facilitate plasticity. However, more detailed mechanisms underlying these processes remain to be uncovered.

The nematode C. elegans is a useful model organism for studying synaptic plasticity due to the simplicity of its nervous system and relative ease of genetic manipulation. The body wall muscle of C. elegans is innervated by cholinergic motor neurons and GABAergic motor neurons, the collaboration of which drives sinusoidal movement of the worm. This coordination depends in part on cholinergic signaling through postsynaptic acetylcholine receptors (AChRs) in GABAergic neurons to produce alternating relaxation or contraction of muscles on opposing sides of the body. The Dorsal D (DD) GABAergic motor neurons of C. elegans undergo extensive synaptic remodeling during development. DD neurons initially receive synaptic input from cholinergic neurons on the dorsal side while innervating ventral muscles. This polarity is reversed at the end of the first larval stage with the presynaptic structures relocating to the dorsal side in exchange for the translocation of postsynaptic components to the ventral side. This synaptic remodeling event is transcriptionally blocked by UNC-55 in the Ventral D (VD) GABAergic motor neurons which normally do not reverse polarity during development. Interestingly, while previous studies focused on the presynaptic components of remodeling DD, postsynaptic remodeling is not well understood. This article will review different aspects of AChRs, ranging from receptor assembly, trafficking to synaptic localization in both mammals and C. elegans, with the aim of providing background information for understanding postsynaptic remodeling in GABAergic motor neurons.

AChR subtypes in mammals and C. elegans.

Two types of AChRs, muscarinic AChRs (mA-ChRs) and nicotinic AChRs (nAChRs or iAChRs) are currently known based on their sensitivity to the agonists, muscarine and nicotine, respectively. mAChRs are G-protein coupled receptors that mediate slow metabolic responses via second messengers, whereas nAChRs are members of the Cys-loop family of ligand-gated ion channels that effect rapid changes in membrane potential. Both receptor types are widely expressed in the nervous system and are linked to
a variety of neuronal functions.

Seventeen nAChR subunit genes are currently characterized in mammals. They encode nAChR subunits with similar transmembrane topology. Each nAChR subunit contains a large extracellular N-terminal domain, which is responsible for ligand binding, followed by four hydrophobic transmembrane regions (M1 to M4) and finally, a short extracellular C-terminus. The cation-selective nAChRs contain a total of five subunits, which are either homomeric or heteromeric. The diversity of nAChR subtypes and their trafficking to either dendritic, somatic, axonal, presynaptic or postsynaptic locations contribute to the various roles that these receptors play in the nervous system. Presynaptic and preterminal nicotinic receptors act to increase the release of other neurotransmitters, whereas postsynaptic and non-synaptic nAChRs mediate excitation as well as activity-dependent modulation of circuits and intracellular enzymatic processes. Abnormal nAChR function is currently associated with certain neurological diseases, including Alzheimer’s Disease and Parkinson’s Disease. Quite interestingly, the wiring pattern of cholinergic input to GABAergic neurons in C. elegans is also present in the mammalian central nervous system (CNS). GABAergic interneurons play an important role in controlling circuit activity in the hippocampus where α7 nAChRs respond to signals from cholinergic neurons, which in turn inhibits nearby pyramid neurons. nAChR activation also directly potentiates glutamatergic neurons, adding to the plasticity of the nervous system.

Figure 1. The motor circuits in C. elegans. (a) AChR compositions in cholinergic motor neuron, GABAergic motor neuron and muscle cells. (b) Agrin/MuSK signaling in clustering of AChRs in the NMJ. Figure is modified from Philbrooke et al. (2013), with permission from the authors.

At least 120 of the 302 neurons in C. elegans nervous system are cholinergic, and AChR subunits can be expressed in both neurons and muscle cells. To date, 29 iAChR encoding genes are currently identified in C. elegans. iAChRs in the neuromuscular junction (NMJ) of the C. elegans have been divided into L-AChRs and N-AChRs based on their sensitivity to levamisole and nicotine, respectively. The L-AChRs are heteropentamers composed of three α subunits, UNC-38, UNC-63, LEV-828–30, and two non-α subunits, LEV-1, UNC-2924. In contrast, N-AChRs in the muscle are homopentamers composed of ACR-16 subunits or heteropentameric αβ-type receptors31,32. “The AChR subunit compositions of C. elegans neurons are less well-studied. Jospin, Barbagallo and colleagues independently reported that ACR-2, ACR-3, UNC-38, UNC-63 and ACR-12 function in cholinergic neurons33,34. These receptors are localized extrasynaptically where they are proposed to function as modulators of cholinergic neuron excitability. Although the full complement of nAChR subunits that function in GABAergic neurons remains unknown, a recent study ACR-12 localization in GABAergic neurons that are localized opposite to synaptic vesicles of cholinergic motor neurons and is thus likely to function postsynaptically35. In addition, acr-12 mutants showed locomotor defects consistent with a postsynaptic role in GABAergic neurons of regulating motor circuit function35.

AChR assembly, synaptic targeting and elimination

The vertebrate NMJ serves as a valuable model in studying the biosynthesis and trafficking of AChRs. Five nAChR subunits are expressed in the skeletal muscle cells (α1, β1, γ, δ and ε), with one subunit (γ to ε) differing between the embryonic and mature muscle cell. After synthesis in the endoplasmic reticulum (ER), nAChR subunits are subjected to a series of post-translation modifications, including glycosylation, disulfide-bond formation and palmitoylation which are crucial for normal function36–37. Before AChRs are exported from the ER, they are folded into pentameric complexes in a fixed stoichiometry. The M3-M4 cytoplasmic loop is required in this compartment for proper subunit folding, cell-surface expression and receptor targeting. AChRs can also be transcriptionally regulated by neuregulin-1 (Nrg1) and neuregulin-2 within the few muscle nuclei that are at the postsynaptic site, leading to the formation of a focal and highly specialized synaptic site. Nrg-1 triggers a signaling cascade of kinases such as Ras, Raf, MAP kinases by activating ErbBs. The activation of these signals in turn enhances transcription of AChR genes by binding to members of the Ets family of transcription factors, GABPα and GABPβ.

In the NMJ, AChRs are pre-patterned at the end-plate region before innervation due to activation of a muscle-specific receptor tyrosine kinase, MuSK, which occurs in an agrin-independent manner. Further activation of MuSK by agrin upon motor axon innervation leads
to the phosphorylation of the β subunit of AChRs and the redistribution of AChRs to the synaptic contact site together with another cytoskeletal protein, rapsyn. Surprisingly, agrin was found not to directly interact with MuSK. It wasn’t until several years ago that Kim et al. and Zhang et al. identified that Lrp4, a member of the lipoprotein receptor-related protein family, was an indispensable co-receptor of agrin in MuSK activation. Later studies revealed that another muscle-specific adaptor protein, Dok-7 is also essential in the above processes of properly clustering of AChRs at the NMJ. A large number of additional molecules and pathways downstream of MuSK activation are also present, such as Rho GTPase, PI 3-kinase, etc, which are reviewed in a great detail by Wu et al.

Studies of AChRs in *C. elegans* NMJ also provide extensive information for understanding AChR biology. Interestingly, agrin is not required for NMJ formation and AChR clustering in *C. elegans*. However, two other chaperone proteins, RIC-3 and UNC-50, first identified in *C. elegans*, are essential for the maturation of AChRs and cell surface expression in mammals. Additionally, a muscle secreted protein, LEV-9 and a transmembrane protein LEV-10, identified in *C. elegans* as ancillary proteins, are required for synaptic membrane localization of L-AChRs. MOLO-1 was the first novel auxiliary protein discovered for Cys-loop receptors that affects AChR function. In addition, a recent article reported that an ADAMTS-like protein, MAD4-A, secreted by cholinergic neurons is required for the clustering of AChRs at cholinergic NMJ. Interestingly, the short isoform of the protein, MAD4-B, expressed by GABAergic neurons, is able to facilitate the clustering of GABA receptors to the GABAergic NMJ, suggesting a universal role of the protein in clustering Cys-loop receptors at the NMJ.

Compared with the comprehensive understanding of AChR assembly and function at the NMJ, mechanisms of cholinergic synaptogenesis in the CNS are largely elusive. While basic mechanisms of AChRs biosynthesis, folding, assembly and trafficking are quite similar, neuronal AChRs do have their own features. For example, homomeric α7 AChR is one of the most abundant AChRs in the CNS, with highest expression level in the hippocampus. Brain-derived neurotrophic factor and tyrosine de-phosphorylation of AChR subunits are thought to facilitate the up-regulation of cell-surface α7 nAChR. Additionally, SNARE-dependent trafficking is also critical for α7 nAChR cell-surface expression in the CNS.

The removal of AChRs from the postsynaptic membrane is another important aspect of postsynaptic plasticity. Synapses with relatively low activity are removed by endocytosis signals emitted from neighboring active synapses. Additionally, endocytosis plays a critical role in this process by affecting AChRs and their accessory proteins in mechanisms that are regulated by intracellular calcium and CaMKII activity. Inactivation of PKA or stimulation of PKC significantly increases the removal of postsynaptic AChRs and depresses AChR recycling. Finally, evidence suggests that Caspase-3, the effector protease involved in apoptosis also mediates the elimination of AChRs by cleaving the WNT signaling protein, Dvl.

### Positive and Negative modulation of Wnt signaling in AChR Clustering

Wnt proteins are a group of highly conserved, secreted lipo-glycoproteins that play profound roles in cell patterning and embryonic development in all animal species. Recent studies reveal key roles for Wnt signaling in nervous system development and function. Three Wnt proteins (i.e. Wnt7a, Wnt5a and Wnt7b) are thought to be involved in general postsynaptic formation in the CNS. These Wnt proteins act post-synaptically by promoting PSD95 clustering and CaMKII activation. While the role of Wnt in clustering AChRs in the CNS is not well understood, extensive research suggests important roles for Wnt signaling in AChR clustering during NMJ development. In vertebrates, Wnt signaling act as both positive and negative regulators for NMJ development. At least two Wnts, muscle-derived Wnt3 and non-muscle Wnt11, appear to activate the formation of preclusters of AChRs through non-canonical pathways, and subsequently promote the expansion and stabilization of preclusters into mature AChRs in an agrin-dependent manner. In contrast, Wnt3a induces the dispersal of AChR clusters by down-regulating rapson expression in a canonical pathway involving β-catenin, suggesting a balance of positive and negative signaling pathways is present during NMJ development, and thus providing a sophisticated regulatory mechanism for precise placement of synapses. Work in Drosophila also contributes useful information regarding AChR clustering at the NMJ. However, due to limited space for this review, please see reviews by Koles and Budnik for further information.

The first Wnt characterized to function in *C. elegans* NMJ formation was Lin-44/Wnt, which is secreted by epidermal cells to restrict pre-synaptic assembly. A recent study suggests that neuronally released CWN-2 could bind with CAM-1/Lin-17 heteromeric receptor, leading to an increase in postsynaptic muscle membrane insertion of ACR-16. Additionally, CWN-2 release depends on neuronal activity, thus providing a novel link between activity-dependent Wnt signaling and postsynaptic nAChR membrane localization. However, how Wnt signaling is
involved in GABAergic neuron postsynaptic remodeling (i.e., ACR-12 trafficking) remains unknown, but inferring from our current understanding of both mammalian and *C. elegans* NMJ assembly, it could involve a complex set of Wnt signals that regulate key steps in the process in anterograde, retrograde and autocrine manners.

**IgSF proteins in postsynaptic AChR localization**

The Immunoglobulin superfamily (IgSF) includes a large group of membrane proteins that share a similar structural domain known as the immunoglobulin domain. IgSF proteins are commonly associated with roles in the immune system, however, recent studies in both vertebrates and invertebrates demonstrate that IgSF proteins are involved in many aspects of neuronal development, including cell and axon migration, synapse formation and function. Many IgSF proteins act as either homo- or heterophilic cell adhesion molecules (CAMs) including neural cell adhesion molecules (NCAMs), whereas other IgSF proteins act as secreted ligands, or auxiliary subunits that facilitate the normal function of specific receptors. Examples of NCAMs are neurexins and neuroligin, the interaction of which induces the formation of both pre- and postsynaptic compartments. A recent study found that an IgSF protein, IgSF9, promotes inhibitory, but not excitatory, synapse development in CA1 pyramidal neurons independent of its cytoplasmic tail, presumably by its CAM-like activity. Another independent study determined that a closely related IgSF protein, IgSF9b, is also required for inhibitory synapses formation on the postsynaptic side. However, this facilitation is not through its direct clustering effect of GABA_A receptors, but rather through its intracellular interaction with neuroligin 2, via the multi-PDZ protein S-SCAM. These studies suggest that IgSF proteins can act in a variety of ways to regulate synaptic plasticity.

IgSF proteins are also expressed in *C. elegans*. Their roles in shaping the nervous system, especially in clustering postsynaptic receptors, are becoming evident. Despite their structural similarities, IgSF proteins have distinct roles in postsynaptic formation and maintenance depending on their localization and molecular structure. An RNAi screen of cell surface Ig-domain-containing proteins for roles in cholinergic synaptic transmission, revealed that RIG-3, a pre-synaptically expressed, GPI-anchored IgSF protein, negatively regulates ACR-16 clustering at the postsynaptic membrane. Interestingly, this RIG-3 dependent function opposes the positive role of Wnt signaling through the Ror receptor, CAM-1, in promoting ACR-16 receptor assembly. These data suggest that the IgSF protein RIG-3 antagonizes the membrane insertion of ACR-16, and thus plays an important role in refining postsynaptic components in this synaptic circuit.

In other studies, Rapti et al. found that another IgSF protein, OIG-4, is critical for L-AChR synaptic localization and function as well. Unlike RIG-3, which functions via its extracellular interaction with Wnt proteins in preventing activity-induced changes in postsynaptic receptor fields, OIG-4 is a muscle-secreted protein that directly interacts with the L-AChR/LEV-9/LEV-10 complex and is essential for clustering these receptors. A homolog of OIG-4, OIG-1 is highly expressed in GABAergic motor neurons. Unpublished work from the Hobert lab at Columbia suggests that OIG-1 exerts a negative effect on presynaptic remodeling of DD motor neurons. Given the structural similarity of OIG-1 to OIG-4 and its strong expression in GABAergic neurons, we tested OIG-1 for a role in postsynaptic assembly and determined that it strongly inhibits remodeling of the postsynaptic ACR-12 in larval GABAergic neurons.

**Concluding remarks**

Synaptic plasticity is critically important for learning and memory formation. While research in the past two decades advanced our understanding of postsynaptic plasticity, the underlying mechanisms that drive plasticity are not well understood. Many questions remain to be answered including: What are the mechanisms that control neurotransmitter receptors biosynthesis? How do receptors traffic into and out of the postsynaptic compartment? How does postsynaptic plasticity affect behavior? New technologies such as advanced imaging technologies, optogenetics, CRISPR/Cas-9 genomic editing are bringing research of neural plasticity into a whole new level. Additionally, studies of postsynaptic remodeling of AChRs in *C. elegans* GABAergic motor neurons could substantially enhance our understanding of neural plasticity and thus shed light on potential therapeutics for neurological and cognitive disorders.

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Drugs of abuse remain a significant burden on society. In addition to direct costs on healthcare and the economy, there are indirect effects on family and loved ones. Addictive drugs alter the nucleus accumbens (NAc), a key region within the brain’s reward system. It is thought that synaptic changes in the NAc mediate drug-related behavior. Though functional remodeling of synapses are traditionally thought of as a neuronally-regulated phenomena, evidence from other brain regions suggests that the innate immune system also plays a role in causing synaptic change. In discussing these topics, this review is divided into 3 parts: 1) an overview of mechanisms underlying NAc synaptic changes in relation to drug exposure, 2) how microglia, a component of the brain’s innate immune system, mediates synaptic change, and 3) a summary of evidence suggesting an interplay between the brain’s reward system and innate immune system. A better understanding of reward system function may provide insight into new therapeutic targets to combat drug reward and addiction.

**Keywords**  microglia, nucleus accumbens, drug abuse, reward system

**Introduction**

Illicit drug use and abuse remains a substantial problem affecting 9.2% of the population aged 12 and over and costs the United States $193 billion per year. The development of drug dependence and addiction is thought to occur through substances commandeering neural pathways involved in processing “natural” rewards such as food or sex. The nucleus accumbens (NAc) is a key brain region involved in drug-related behavior. Normally acting as a region transforming motivation to goal-directed actions, the NAc is a structure altered by virtually all drugs of abuse. Drug-induced changes in synaptic physiology of this region are thought to be a causal step in the development of subsequent drug-related behaviors.

With the goal of better-understanding drug addiction, the majority of current research is devoted to a region-specific, neuron-centric examination of drug effects. Though this approach revealed many important insights on mechanisms of synaptic plasticity relating to drug use, there is growing evidence from other brain regions pointing to the possibility of microglia, a component of the brain’s innate immune system, mediating synaptic change. This review will give a brief summary of drug-induced synaptic changes occurring in the NAc in relation to behavior followed by an overview of how microglia in the context of the neuroimmune system may be involved in some of these changes.

**A kNA.k for drug-induced changes: overview of structure and mechanisms of plasticity in the accumbens**

The NAc is a structure within the ventral striatum that integrates afferents from cortical and limbic regions. It is divided into 2 regions—the NAc shell and NAc core. The latter is continuous with the dorsal striatum. As a whole, the NAc receives glutamatergic inputs encoding emotional valence from the basolateral amygdala (BLA), contextual relevance from the ventral hippocampus (vHipp), and action/outcome reinforcement information from the medial prefrontal cortex (mPFC). The NAc also receives inputs from several thalamic regions as well as modulatory inputs from the lateral hypothalamus, midbrain, and brainstem. The majority (approximately 90%) of neurons found within the NAc are gamma-aminobutyric acid (GABA) releasing medium spiny neurons (MSN). The remainder are GABAergic or cholinergic interneurons. The MSNs are further divided into those expressing D1 dopamine receptors projecting primarily to midbrain dopamine areas to form the “direct” pathway and those expressing D2 receptors projecting to the ventral pallidum to form the “indirect” pathway (Figure 1). The direct/D1 MSN pathway of the NAc is thought to enhance the rewarding effects of drugs of abuse whereas the indirect/D2 MSN pathway is thought to

a. Addiction: compulsive use of a drug despite the negative consequences.

b. Ventral striatum: An anatomical region consisting of the NAc and olfactory tubercle.

c. Dorsal striatum: An anatomical region made up of the caudate and putamen. These structures play a role in the extrapyramidal motor system.
Synaptic plasticity in the NAc

Synaptic plasticity is a widely accepted mechanism for learning and memory. Over the last few decades, researchers have demonstrated that forms of both Hebbian plasticity and homeostatic plasticity occur within the NAc. Low frequency electrical stimulation of the NAc shell causes NMDA receptor (NMDAR; 1 Hz or 5 Hz)- or metabotropic glutamate receptor (mGluR; 10 Hz or 13 Hz)-dependent long-term depression (LTD). In contrast, high frequency stimulation (HFS; 100 Hz) of the NAc core causes long-term potentiation (LTP) dependent on Mitogen-activated protein kinase kinase (MEK) and N-methyl-D-aspartate receptors (NMDARs). Lastly, research performed using NAc cultures show that these MSNs undergo bidirectional synaptic scaling. It was recently demonstrated that this effect may be mediated by brain derived neurotrophic factor (BDNF).

Perhaps more interesting is the interplay between synaptic changes in the NAc and drugs of abuse in modulating behavior. In mice, repeated cocaine administration causes locomotor sensitization (a model of drug induced learning and memory) and re-exposure following withdrawal occludes NMDAR-dependent LTD. Preventing clathrin-mediated endocytosis of AMPA receptors (AMPA) blocks LTD induction and behavioral sensitization to amphetamine. With the advent of bacterial-artificial chromosome (BAC) transgenic mice expressing fluorescent proteins under D1 or D2 receptor promoters, investigators began examining NAc synaptic properties and behavior in an output/pathway-specific manner. Grueter et al. examined D1 and D2 MSNs in the NAc core of mice and found that a 10 Hz electrical stimulation causes a metabotropic glutamate receptor (mGluR) dependent LTD in D2 MSNs. Furthermore, these investigators demonstrated that this mGluR LTD is dependent on presynaptic cannabinoid receptor 1 (CB1) and postsynaptic transient vanilloid type 1 (TRPV1) function and is abolished 24h post-cocaine exposure. This same research group also found that upregulation of the transcription factor ΔFosB enhanced cocaine-induced behaviors. This was associated with an increase in silent synapses and spine density in D1 MSNs of the NAc shell. Overall, these studies suggest that cocaine affects NAc synaptic plasticity at multiple levels ranging from neuromodulators to transcriptional and structural changes. Interestingly, cocaine exposure also causes downregulation of cysteine-glutamate exchange and decreases extracellular glutamate levels in the NAc. This combined with the observation that restoring basal cysteine-glutamate exchange abolishes cocaine seeking/reinstatement behavior in rodents suggests that drug addiction is caused in part by dysregulation of glutamate homeostasis.

Relating plasticity to behavior

The development and optimization of optogenetics permits examination of NAc physiology in an input-specific manner. Through viral infection of channelrhodopsin (ChR2) into the mPFC, BLA, or vHipp followed by light-stimulation of axon terminals in the NAc, investigators began teasing apart afferent-specific synaptic changes. This method first demonstrated that increased excitatory drive from any of the 3 glutamatergic inputs alone (mPFC, vHipp, or BLA) provides a rewarding stimulus to drive goal-directed behavior. Following the observation that a single exposure to cocaine occludes HFS-induced LTD of NAc shell D1 MSNs, Pascoli et al. showed that NMDAR-dependent LTD of mPFC inputs onto the NAc shell in vivo abolished cocaine-induced locomotor sensitization. More recently, this same group used viral infections of ChR2 in BAC transgenic mice to examine input-output-specific synaptic alterations of the NAc shell MSNs following a month of withdrawal from cocaine self-administration. After establishing that cocaine withdrawal causes AMPAR rectification and increased the AMPA/NMDA ratio (A/N) in only D1 MSNs, they found that NMDAR-dependent LTD on vHipp inputs in vivo normalized the A/N and reduced seeking vigor during cue associated cocaine seeking. Additionally, mGluR-dependent LTD on vHipp inputs in vivo normalized rectification and reduced seeking discrimination. Finally, Pascoli et al. found that mGluR-dependent LTD on mPFC inputs in vivo normalized both rectification and A/N while completely abolishing drug seeking behavior. Curi-

### Glossary

- **Hebbian plasticity**: Changes in synaptic strength due to one neuron firing and signaling to another neuron.
- **Homeostatic plasticity**: Circuit-wide, activity-dependent changes in synaptic strength. This acts to stabilize neuronal activity in the context of perturbations.
- **Long term depression (LTD)**: Activity-dependent weakening of synaptic transmission lasting at least an hour.
- **Long-term potentiation (LTP)**: Activity-dependent strengthening of synaptic transmission lasting at least a hour.
- **Synaptic scaling**: Decreased or increased surface AMPAR expression resulting from prolonged activity increase or blockade, respectively.
- **Silent synapse**: A synapse made up exclusively of NMDAR.
ously, they did not find any changes in BLA-specific inputs onto the NAc with cocaine withdrawal. This contrasts with another study in rats where prolonged withdrawal following cocaine self-administration caused an increase in silent synapses followed by an increase in AMPAR rectification in BLA specific inputs onto the NAc shell\(^22\). It will be interesting to see how future studies resolve this discrepancy.

Microglial effects on macroscopic behavior: lessons from the hippocampus and beyond

Microglia are one of several macrophages of the central nervous system\(^23,24\) (CNS). These cells make up 10% of the brain parenchyma\(^25\) and play a key role in mediating immune responses in this region\(^25,26\). Under basal conditions, these cells adopt a “ramified” morphology consisting of a small soma and fine, motile cellular processes\(^27\). These processes scan the neuronal environment. Upon detection of altered CNS homeostasis including infections, ischemia, and altered neuronal activity, microglia lose these fine processes and take on an amoeboid, “activated” morphology with an altered gene expression profile\(^27\). Both ramified and amoeboid microglia exhibit phagocytic activity\(^25\).

In addition to mediating immunologic responses, microglia modulate synaptic activity through physical and chemical interactions with neurons. Facial nerve injury\(^25\) or cortical injection of bacterial adjuvants\(^28\) induce synaptic stripping—where local activation of microglia results in the physical removal of synaptic inputs on the regional neurons\(^25\).

**Microglia talking with neurons: discussion on the synapse and behavior**

One of the mechanisms by which microglia communicate with neurons to modulate synaptic physiology is through the use of the fractalkine/CX3CL1. This is a chemotactic cytokine\(^1\) constitutively expressed by forebrain neurons including the striatum\(^29\). In the CNS, microglia are the only cells known to express the fractalkine receptor, the \(G_i/G_o\)-coupled receptor CX3CR1\(^29\). In acute hippocampal slices, one research group found that bath application of fractalkine prevents induction of HFS LTP in CA1 pyramidal cells in an adenosine receptor-3 and CX3CR1-dependent manner\(^30,31\). Interestingly, a separate research group found that hippocampal CA1 HFS LTP could not be induced in CX3CR1 knockout mice\(^32\). Though methodological differences between these studies may account for the discrepancy in results, it’s worth noting that both sets of researchers found hippocampal synaptic plasticity altered through perturbing a microglial signaling cascade.

In addition to direct interactions between neurons and their phagocytic neighbors, microglia also secrete a variety of chemical factors known to influence synaptic physiology\(^27\). Both tumor-necrosis factor-\(\alpha\) (TNF\(\alpha\))\(^33\) and BDNF\(^34\)
are released by microglia and are known mediators of synaptic scaling (Figure 1). Microglial BDNF also regulates the chloride reversal potential in spinal cord neurons.

More recently, investigators examined synaptic and behavioral effects of knocking out microglia. Parkhurst et al. created an elegant microglia-specific conditional knockout mouse to assess these cells’ function independent of development. These investigators found that microglial elimination in adult mice leads to behavioral deficits and synaptic changes. Specifically, these mice show a decreased fear conditioning response, decreased novel object recognition, and decreased performance-based improvements on the rotorod. At the cellular/synaptic level, these mice exhibited decreased spine formation, decreased AMPAR and NMDAR-dependent miniature excitatory post-synaptic current (mEPSC) frequency, and decreased BDNF mRNA. This same study also found that knocking out microglial BDNF is sufficient to induce the deficits in fear conditioning and rotorod performance.

Additional evidence pointing towards the importance of microglia in regulating synaptic physiology comes from neuronal changes observed as a result of exogenously activating microglia. Pascual et al. used lipopolysaccharide (LPS), an agonist of toll-like-receptor-4 (TLR4), to activate microglia and assess changes in hippocampal synaptic physiology. These researchers found that LPS bath application on acute mouse brain slices resulted in a temporary increase in spontaneous EPSC frequency of hippocampal CA1 pyramidal cells. This phenomenon was found to depend on TLR4 expression, mGluR5 expression, and microglial ATP release acting on astrocytes. Interestingly, another group of investigators found LPS combined with hypoxia induces longer-lasting changes in the form of LTD in hippocampal CA1 pyramidal cells dependent on complement receptor 3 (CR3) but not TLR4. This change was associated with a decrease in mEPSC amplitude but not frequency suggesting a postsynaptic change. Behaviorally, peripheral LPS injections result in decreased food intake/weight, decreased social behavior, anhedonia, peripheral inflammation, and neural inflammation. The synaptic and behavioral changes occurring as a direct result of microglial perturbation demonstrates the importance of this cell population in regulating neuronal transmission.

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Excitatory post-synaptic current (EPSC): Glutamatergic currents measured from a single neuron. Mini-EPSCs are measured in the presence of tetrodotoxin to assess glutamatergic activity in the absence of action potentials. Spontaneous EPSCs assess the glutamatergic activity in the presence of action potentials.

Anhedonia: Inability to experience pleasure from previously rewarding stimuli.

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Facing the drug MeNA e: connecting the neuro-immune system to drug-related changes

The literature surrounding the cellular basis of drug addiction centers on neuronal changes in the NAc. Research examining the effects of microglia and the immune system on synaptic transmission is largely focused on the spinal cord or hippocampus. As of now, no study has explored the hypothesis that microglia are necessary for the drug-induced behavior and synaptic changes observed on NAc MSNs. However, there are several pieces of evidence hinting that this interaction exists and takes place in a bidirectional manner.

On one hand, neuronal activity influences microglia and inflammatory molecules in the CNS. Seizures induced with kainic acid result in cytokines released in affected regions. In the spinal cord, stimulation of sensory afferents results in an increase in activated microglial markers. Drugs of abuse such as cocaine alter activity in the NAc of rats, mice, non-human primates, and humans. On the other hand, inflammatory molecules also influence neuronal function. In addition to TNFα mediating synaptic scaling as mentioned above, interleukin (IL)-6, IL-1B, and interferon-gamma are also implicated in mediating hippocampal synaptic physiology.

Another set of studies show direct interaction between drugs of abuse and the neuro-immune system. The opioids morphine and remifentanil were found to bind with TLR4 and its accessory protein, myeloid differentiation factor 2 (MD2). Furthermore, pharmacologic block or knocking out TLR4 reduced morphine conditioned place preference and dampened morphine-induced increases in NAc extracellular dopamine levels. Although TLR4 expression is typically thought limited to microglia, there is some evidence suggesting that this receptor is also expressed on neurons. TLR4 is also activated with ethanol and plays a role in alcohol-induced brain damage, inflammation, and behavioral changes in mice. Given the different behavioral responses elicited with opioids and ethanol compared to sickness and anhedonia induced by LPS, it will be interesting to see how TLR4 signaling causes these effects. Future studies may also resolve the discrepancy of where TLR4 is expressed as well as test whether other illicit drugs such as cocaine bind to TLR4/MD2.

In addition to TLR4, microglia express a large variety of proteins and receptors previously studied as regul-
tors of neuronal function. CB1 and TRPV1, mediators of mGluR-dependent LTD in the NAc and hippocampus are both expressed on microglia. Interestingly, within the peripheral immune system, CB1 expression is necessary to generate a TLR4 response. Microglia also express NMDAR—a key player in the eponymously-named. This suggests that similar to neurons, there are region-specific differences in microglia. The function of microglial TRPV1, CB1, NMDAR, D1, and D2 receptor agonism in the context of normal and drug-induced physiology remains to be determined.

Summary

Illicit drug use and abuse remains a substantial burden to society. The last decade of research revealed that changes in NAc MSN synaptic physiology mediates some of the behavioral manifestations of drug exposure/addiction. More tellingly, reversal of drug-induced synaptic changes in this region mitigates or reverses drug-mediated behaviors. Meanwhile, research in other regions of the CNS point towards the importance of microglia and the immune system in mediating synaptic physiology and behavior. Though there is some evidence in the context of adolescent drug exposure resulting in altered adult TLR4 expression in the NAc, there have been no studies examining the role of microglia and neuro-immune system modulators on NAc MSN synaptic physiology. Future studies need to combine techniques used to study hippocampal and spinal cord microglial/immune system effects with drug-induced input and output-specific electrophysiology used in recent NAc studies. Such an endeavor will better characterize changes occurring in the drug-altered brain and may provide novel therapeutic targets.

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The Activation of Heterotrimeric G Proteins: Evaluation of Past and Present Mechanistic Models

Alyssa Lokits

Cell signaling through G protein coupled receptors (GPCRs) represents one of the most modulatory and regulatory communicative mechanisms in the nervous system. Extracellular signals in the form of neural peptides, hormones, neurotransmitters, small molecules, and ligands are converted into neural responses through GPCR interactions with heterotrimeric G proteins. Since their discovery, GPCRs have become the largest therapeutic target for a wide range of human pathologies including diseases such as Schizophrenia, autism, neuropathic pain, sleep/wake disorders, and bipolar disorder. However, despite their importance, the mechanism of G protein activation through their cognate receptors for signal transduction has not been fully elucidated. This review looks to introduce the basic structure and function of heterotrimeric G proteins, the challenges present in the field which have hindered our understanding, and summarize the different proposed mechanisms of activation leading to intracellular signaling cascades. Lastly, our current working model of activation will be presented as a unified mode of understanding these dynamics while predicting where the field looks for future drug discovery and research.

Keywords  GPCRs, signal transduction, structural biology

Introduction

Cell signaling is a fundamental process required in all living organisms for development and homeostasis. In neurons, rapid cell to cell signaling via chemical synapses requires ligand-gated ion channels, which quickly activate and alter the membrane potential through their ability to directly fluctuate local ion concentrations\(^1\). Slower or more modulatory signaling is often mediated through G protein coupled receptors (GPCRs), which require more time to alter the membrane potential as they are not ion channels themselves, but interact with transducing proteins to communicate their signal and propagate an intracellular response\(^2\). GPCR-mediated ion channels along the post synaptic membrane are therefore acted upon through a course of protein-protein interactions. With around 800 different types of GPCRs, this modulatory class of cellular receptors accounts for 2% of the expressed protein population\(^3\). This diversity of receptor subtype provides an additional layer of neuron and synapse specific signaling.

GPCRs are the largest and most diverse class of membrane receptors in eukaryotes\(^4\). In the nervous system, GPCRs can be present both pre-and post-synaptically to differentially influence neuronal communication\(^5\-\(^8\). This allows for modulation on all sides of the “message” being transmitted. Likewise, the ability of GPCRs to bind many different forms of ligands makes these receptors responsible for an estimated 40% of all signaling within the cell\(^9\). This significant role in cell to cell communication in conjunction with their transmembrane locations makes GPCRs a prominent therapeutic target\(^10\). Though roughly half of current therapeutics act upon GPCRs, a consensus model of the mechanism of G protein activation via their receptors does not exist. Such a model would provide better understanding of a modulator of neuronal communication as well as increase the current understanding of cell signaling on a broader scale.

G Protein Signaling Cycle

As implied by their name, GPCRs interact with intracellular, heterotrimeric complexes, called G proteins, in order to transduce their signal into a cellular response. This signal exchange occurs, as depicted in Figure 1 with the rhodopsin GPCR, when an external ligand activates the receptor, allowing for association of the heterotrimer. Composed of three subunits, the G\(\alpha\) and functional G\(\beta\)\(\gamma\) dimer subunits become activated after binding to an activated receptor (R*); this interaction catalyzes the G\(\alpha\) subunit to exchange GDP for GTP. This requires a conformational change that rearranges the binding affinities within the complex, promoting the G\(\alpha\) subunit to dissociate from the receptor and its G\(\beta\)\(\gamma\) subunits. The activated and dissociated G\(\alpha\) may then interact with and initiate signaling cascades with its effector proteins while in the GTP-bound state. However, as most G\(\alpha\) subunits possess intrinsic enzymatic
activity, the $\gamma$ phosphate of the GTP is ultimately hydrolyzed resulting in a GDP-bound $G\alpha$ subunit. This conformation, once again, maintains a higher affinity for its $\beta\gamma$ pair than its effector proteins, allowing for the termination of the signal and the recycling of the transducer signaling machinery at the membrane interface for future signaling events coupled to the receptor\(^2\,9\,11\). The present work seeks to synthesize the abundance of literature surrounding this activation process and the current mechanistic models of the allosteric interaction networks required to modulate the $G\alpha$ subunit for activation.

**Difficulties to elucidate the Structure and Mechanics**

The ligand binding site in the archetypical, Class A rhodopsin GPCR (required for phototransduction in vision) is approximately 40 Angstroms away from its intracellular loops, which bind to the $G\alpha$ subunit\(^12\,13\,14\). The interface of the receptor-$G$ protein is 30 Angstroms away from the $G\alpha$ subunit’s nucleotide binding pocket\(^8\,11\). Therefore, the extracellular ligand must induce an allosteric conformational change some 70 Angstroms from its binding site; a dynamic conformational change must propagate across the complex to release the basal GDP nucleotide in exchange for GTP. This has led to multiple proposed $G\alpha$ activation mechanisms; each model attempts to understand how the securely lodged nucleotide becomes expelled from within the GTPase and helical domains of $G\alpha$.

However, there are many practical and technical difficulties hindering the elucidation of this mechanism. One problem is the inherent GPCR-$G$ protein cross talk, which makes studying only one $G$ protein subtype difficult. Multiple $G\alpha$ subunits were shown to interact with the same receptor, but elicit a different cellular response\(^15\). Likewise, it is still not understood which of the various possible $\beta\gamma$ combinations\(^16\,17\) and $\alpha/\beta\gamma$ combinations\(^18\,19\) are possible *in vitro* and *in vivo*. Therefore, all mutational studies for $G$ protein activation are severely limited in scope as they only focus on a handful of known $G$ protein $\alpha$, $\beta$, and $\gamma$ combinations. Confounding this limitation in the first messenger signal components is cross talk between the downstream signaling effector molecules activated by $G\alpha$ and $\beta\gamma$. This creates caveats in using indicators such as cAMP accumulation as measures of $G$ protein activation, as it can be activated by both the $G\alpha$ subunit and $\beta\gamma$ subunits of different subfamilies. Technical difficulties with expressing and purifying individual subunits and receptors in large enough quantities for experimentation has led to the use of many different cell lines for the various protein components. Even once these challenges are met, many studies are limited by the presence of endogenous signaling machinery present in the experimental cell lines.

Additionally, crystallization of these proteins in their various states has been a challenging enterprise, as these complexes are composed of highly flexible domains, very transient conformational switching states and a lipid membrane-bound receptor. Even with recent advances in crystallographic techniques, nanodiscs and stabilizing nanobodies, crystal structures only provide a “snapshot” of one or more of the various conformations the protein complexes may undergo\(^20\,21\). Instead, to probe the various physiologically relevant conformations the complex is capable of maintaining across the entire signaling cycle, a more dynamic approach is required to elucidate the modulatory and inherently flexible states these proteins undergo during the signaling cycle.

**Current Structural Knowledge**

The elucidation of atomic resolution crystallographic and NMR structures has been paramount to the advances in conformational dynamics of $G$ protein activation. The GDP-bound crystal structures\(^22\,23\) of the heterotrimeric $G$ protein, as well as the dissociated dimeric subunits of $G\beta\gamma$ complexes\(^24\), were key to understanding the basal, unbound state of the signaling complex. Crystallization of

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**Figure 1** The most studied GPCR, Rhodopsin, becomes activated ($R^*$) by the energy of a single photon. $R^*$ undergoes a change in structure allowing it to bind to its trimer (G$t\beta\gamma$) and catalyze nucleotide exchange via an unknown mechanism. G$t(GTP)$ then dissociates to interact with downstream effectors such as Phosphodiesterase (PDE) which hydrolyzes cGMP to GMP. Decreased levels of cGMP leads to the closure of cGMP-gated Na$^+$ channels. With cations inhibited from entering the photoreceptor, the membrane hyperpolarizes. (adapted from Neuroscience, Purves, 4th Ed.)
the activated Gα subunits in the presence of various GTP analogues provided the structural snapshots of the final stage of activation25-27; likewise, structures of Gα and Gβγ subunits with their downstream effectors28 have also led to many interesting discoveries for protein-protein interfaces, signal propagation, and insights into causes for various disease states29.

Wall and colleagues solved the first structure of a G-protein heterotrimer, which provided the structural basis for Gα–Gβ interactions23. Crystal structures of Gαi_1, Gβ2_γ2 and Gαi_2, Gβ2_γ2 show two sites of interaction for the Gα and Gβ subunits. These include the hydrophobic pocket of the Switch I and II regions as well as a portion of the Gα αN helix22, 23. However there is no crystallographic evidence of any Gα-Gγ interaction. The αN helix (amino terminus of the Gα subunit) is in close proximity to the carboxyl terminal tail of Gγ. The αN helix seems to be unstructured when in the monomeric, GDP-bound state, but it becomes helical when bound to Gβγ. Addition of a lipid modification (myristolation) allows it to maintain a βγ-independent helical nature, associate with the membrane and the activated receptor24, 25.

Biochemical and mutational studies have further mapped areas around this helix thought to be important for receptor interaction and G protein activation30-33. From these studies, the Gα subunit’s amino terminus and carboxyl terminus seem to be crucial for R* interaction30, 34-36. Several studies have mapped regions of interest for conformational propagation from the binding interface of the receptor to the nucleotide binding site. Of these around the quinine ring29, the α5 helix35-39 and the Gβ subunit’s40 were identified as important structural moieties for protein interaction and/or function. Likewise for nucleotide exchange and activation of the Gα subunit, initial interaction with R* requires binding of the βγ subunits to the Gα N helix of Gα-subunits. This interaction of Gα with the βγ subunit results in increased nucleotide exchange. The Gβγ subunits have also been shown to provide receptor selectivity with their interaction with Gα50.

Proposed Mechanism of Activation- the Lever Arm

It was hypothesized that the activated receptor must act “at a distance” to induce nucleotide exchange based on the aforementioned crystallographic knowledge of GPCRs and G proteins. Looking at monomeric G proteins of the Ras or elongation factor subfamilies related to the trimeric complex has provided the foundation of insight into a means of disrupting nucleotide stability in the presence of their exchange factors. Though crystallization of the R*-Gαi_1, Gβ2_γ2 had not been solved in 1998, the 3D structure of bacterial elongation factors Tu and Ts in the empty nucleotide transition state had been solved41, 42. From these structures the Bourne lab developed the lever arm model of Gα activation based on the Tu/Ts interface and its similarity to the Gα-Gβ interface22.

From these structural comparisons, they proposed that similar to the three-pronged “comprehensive attack”, the EF-Ts imbed upon EF-Tu for nucleotide destabilization, the Gβ subunit’s action on the binding pocket may be analogous. This included directly expelling the magnesium ion from the pocket, interrupting the loops around this region, and destabilizing the guanine ring of GDP. The overall structural means of catalysis was through the rotation, or tilting, of the Gβ subunit relative to the Gα inter-domain interface. This alteration forces the “lip” occluding the nucleotide pocket to open, creating an exit route59.

Similar to the EF-Tu/EF-Ts mechanism, the lever arm hypothesis also included the rotation of loop and secondary structure elements around the binding pocket in a molecular “tug-of-war” for the binding of GTP versus re-coupling to the Gβ subunit. The rotation of Gα’s α2 helix is consistent with the basal and activated Gα crystal structures, as well as kinetic studies evaluating the affinity and irreversibility of the creation and termination of the stable ternary complex53.

The lever arm model was qualitatively supported using mutant Gα-Gβ with an altered conformation of interaction54. These experiments showed that shortening of the αN helix of Gα by four residues near its amino terminus resulted in increased spontaneous nucleotide exchange by Gβγ; this was thought to be due to a tilted interface between the subunits which altered the exit route “lip” interaction of Gβ resulting in increased nucleotide exchange. Likewise, further biochemical studies mutating residues along the Gα-Gβ interface showed increased instability of this region, leading to altered rates of receptor-mediated nucleotide exchange, but not heterotrimer formation55, 19, 44. Studies also support the hypothesis of the αN helix interacting with and moving upon receptor binding51, 45.

Though the lever arm model outlines a potential mechanism of information propagation across the complex for activation, it inherently possesses several flaws. Small GTPases do not have the long αN terminal helix, yet still possess the same level or higher rate of nucleotide exchange3. Small GTPases also do not require the coordi-
nation of a magnesium ion for binding and hydrolysis. Likewise, speculation of the thermo-stability of the complex suggested that pulling of Gβ subunit alone may not be sufficient to force nucleotide exit. Furthermore, the lever arm model did not account for putative Gγ interactions with the receptor and Gα subunit. Reports that mutations to the carboxyl terminus of Gγ were shown to increase receptor-mediated nucleotide exchange resulted in revisions to the lever arm model to account for the subunits role in stability, selectivity and activation.

Proposed Mechanism of Activation- the Gear Shift

To address some of the issues present in the lever arm model, Chabre and colleges modified the activation mechanism to still include the Gβ subunit, but removed the torqueing, or prying, motion of the Gβ subunit. This change was due to the packing differences seen in the Switch II (binding interface of Gα-Gβ) region between GTP- and GDP-bound Gα subunits. In the GDP-bound form, Switch II was loose and allowed for a water-filled channel; however, it was ordered and densely packed upon GTP binding. Likewise, a disordered interface seemed to be important for GDP instability upon Gβ interaction between the Switch I and II elements. Instead of pulling on the Gβ subunit to pry open the pocket and allow nucleotide displacement, Chabre suggests that Gβ rotates closer to the nucleotide binding region during the exchange. This allows the Gγ subunit to interact with the helical domain of the Gα subunit. The amino terminus of the Gγ subunit could then alter and shift the helical domain of the Gα subunit.

The gear-shift model received its name for the presence of three “gears”. These gears are composed of (1) the activated receptors interaction with the carboxyl and amino terminal helices of Gα and the carboxyl terminus of Gγ, (2) the Gβ subunit interacting with the GTP-binding domain of Gα in the basal, GDP-bound state and (3) the Gγ subunit interacting with the helical domain of Gα. These gears cooperate to imbue conformational alterations across the complex to facilitate GDP instability and release. The Gγ subunit acts as the conformational “shifter” as it is suggested to physically coordinate each of the gears to promote helical domain opening.

A major component of this model is the speculative interaction of the Gα helical domain directly with the Gγ subunit. Several studies have suggested that the helical domain must open for nucleotide exchange to occur, and Gα proteins in which the helical domain was entirely removed possess increased spontaneous nucleotide dissociation. Chabre proposed that the Gγ subunit hooks the Gα helical domain subsequent to Gα-Gβ interface tightening. This is not without biochemical validity as several studies suggested Gα and Gγ could interact, though there was no direct crystallographic or NMR data to support such an interface. In the original description of the gear-shift model, the Gγ subunit displaces the helical domain as a “rigid body” away from the nucleotide binding region. This model also suggests that the amino terminus of the Gγ subunit might also play a role in the efficiency of the exchange process by allowing an additional level of specificity with combinatorial subunit composition.

The gear-shift model is similar to the lever arm model in that the Gβ subunits play a pivotal role in inducing activation. However, unlike the lever arm model, the αN helix is proposed to move in the opposite direction to allow for a tightening of packing at the Gα-Gβ interface. Further support for this model came through observation of similarity between the heterotrimeric G protein β subunits and guanine nucleotide exchange factors associated with the monomeric G protein Arf family (subfamily of the monomeric Ras superfamily). In heterotrimeric G proteins, Gβ has been suggested to play a crucial and active role in GDP exchange for GTP. It has been predicted to alter its relative conformation upon GDP release when the ternary complex is in its transient nucleotide free state. This is similar to the mechanism of activation of small monomeric G proteins and their guanine nucleotide exchange factors (GEFs). For their activation, the GEFs of the Arf family of proteins alter the conformation of the Switch II regions. The Gβ subunit could parallel this mechanism of activation by tightening its interaction during the free complex.

Proposed Mechanism of Activation- the Gα Unified Model

Several site-directed spin label studies from the Hubbell and Hamm laboratories investigated the interface between the Gβγ subunits and the Gα’s Switch I, II and αN helix. Though these studies found displacement of the

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b. **The Gear Shift Model**: Hypothesized model to describe the mechanism of nucleotide exchange during G protein activation. Instead of pulling on the Gβ subunit to pry open the pocket and allow nucleotide displacement, Gβ is suggested to rotate closer to the nucleotide binding region during the exchange. This allows the Gγ subunit to interact with the helical domain of the Gα subunit and allow opening.

c. **Site-directed spin labeling**: A technique to introduce a nitroxide spin label into a Cysteine point mutation via a disulfide bond. This spin label allows for structural studies such as EPR/DEER for measurements of environment, solvation, and distance.
Switch regions\(^d\) and disordered loop conformations that destabilized nucleotide binding, their results did not directly align with either the gear-shift or the lever arm models. Instead, these displacements contradicted the larger conformational changes predicted. Likewise, each of the studies suggested that the G\(\gamma\) subunits rotated perpendicular to the membrane as opposed to parallel, in contrast with both of the aforementioned models\(^1\text{–}^6,^42,^51\). Though these data may potentially be due to the introduced cysteine mutations or the spin labels introduced into the system, these studies led to the search for a new model of G\(\alpha\) activation.

Other experimental findings also conflicted with both models. Primarily these studies investigated the G\(\alpha\) carboxyl terminal helix (\(\alpha5\)). This region of the G\(\alpha\) subunit has been shown to interact with activated GPCRs through much mutational, fluorescence, NMR, EPR/DEER and crystallographic structural studies\(^65\text{–}^70\). Composed of an \(\alpha\) helix, this secondary structural element has been shown to move as a rigid body, connecting the receptor to the nucleotide binding domain\(^71\). This conformational change was suggested to be transmitting activation to the binding pocket via G\(\alpha\)'s \(\alpha1\) and \(\beta2/3\) strands\(^37,^72\). Mutations to this helix and the \(\beta6\)-\(\alpha5\) loop led to spontaneous nucleotide release, indicating that this region is important for the signal propagation from the receptor to the GDP binding site\(^37,^38,^73,^75\). Mutational studies and molecular dynamics indicated that the \(\alpha1\) helix, \(\beta2, \beta3\) and \(\beta6\) strands may be the midpoints to transmit the conformational change from the \(\alpha5\) helix to the helical domain\(^31,^37,^72\). Still other studies indicated that mutations along \(\alpha5\) resulted in decreased affinity for GDP and increased spontaneous release\(^74,^75\). Male patients with early onset puberty were also found to have similar \(\alpha5\) mutations in their G\(\alpha\) proteins\(^73\). In summation, these reports suggest that the G\(\alpha\) subunit could act as its own “microdomain” for activation independent of G\(\beta\gamma\) movement.

The \(\alpha5\) helix was also shown to undergo a rotation, insertion and displacement upon receptor interaction, using a five-glycine linker at base of the \(\alpha5\) helix that “decoupled” the receptor from the nucleotide binding pocket resulting in decreased receptor-mediated nucleotide exchange\(^70\). This decoupling linker did not drastically alter the intrinsic basal rate of nucleotide exchange. These results suggested that movement of the \(\alpha5\) helix upon receptor interaction is necessary for propagation of the conformational change from the receptor binding interface to the nucleotide binding pocket\(^76\text{–}^78\). Therefore, the new unified model of G protein activation is founded on the principle that receptor interaction with the \(\alpha5\) helix is sufficient for nucleotide exchange. Work done by the Hamm, Hubbell and Meiler laboratories suggest that perturbing this region leads to interaction of the highly conserved TCAT motif of the \(\beta6\)-\(\alpha5\) loop. This loop directly interacts with guanine ring of GDP allowing for instability in that region\(^70\).

Lastly, recent crystallographic advances in field corroborated the unified model of G\(\alpha\) activation. In 2012, Kobilka and collaborators solved the nucleotide-free ternary complex in which a ligand-bound GPCR was coupled to its cognate heterotrimeric complex during the transition of GDP for GTP exchange\(^21\). In this crystal structure, the gear-shift model was shown to be highly unlikely as the G\(\gamma\) subunit did not interact with the G\(\alpha\) subunit. Likewise, further studies on this complex corroborated the rotation of the \(\alpha5\) helix upon receptor interaction and potential information propagation to the nucleotide binding pocket\(^79\).

### The Mechanism of G protein Activation Impacts Drug Discovery

Understanding the fine-tuned mechanics of the GPCR-G protein system lends itself to more focused drug targets. As GPCRs and G proteins play a “slow” and modulatory role in neuronal signaling, targeting GPCRs through the use of positive and negative allosteric modulators was shown to augment current therapeutic strategies in order to lessen required dosing and/or modulate GPCR function without direct agonism/antagonism. Strong support for the utility of GPCR modulators in attenuating vagrant signaling cascades can be seen in a range of neurological dysfunctions. Research on diseases such as Parkinson’s\(^80\), addiction\(^81\), psychosis\(^82\), Fragile X Syndrome\(^83\) and memory deficits\(^84\) are all turning to modulators of GPCRs for better medicinal or research compounds.

Roughly 30–40% of approved drugs currently on the market bind extracellularly to G protein coupled receptors\(^10\). However, understanding GPCR-G protein interactions and their mechanics can lead to novel intracellular medicinal targets. With 800 different GPCRs encoded in the human genome and 21 G\(\alpha\) genes\(^8\), creating small molecules which enhance or inhibit GPCR-G protein interaction may provide a novel means of directing cell signaling in disrupted neural circuits. Enhancing or attenuating receptor-G protein specific intracellular dynamics could shift erroneous receptor conformations or over-activated signaling pathways.

Developing interaction-specific GPCR-G protein signaling modulators may also open doors for treatments which synergize with current neurological therapeutics. As many GPCRs couple to multiple G\(\alpha\) subtypes, preferentially
Future Studies of G protein Structure and Dynamics

Though the unified model is not fully tested and is not expected to completely explain G protein activation, it is congruent with the current literature in the field. Furthermore, the unified Gα model also allows for other “routes” of information flow across the Gα subunit to the nucleotide pocket. For example, the molecular trigger model suggests that a conserved binding site between the α5 and β2/3 loop (ARG in the conserved DRY motif) is responsible for the Gα family’s conformational changes upon R* binding. This model utilizes a different “door” or direction of information flow across the Gα subunit, connecting the receptor to the nucleotide binding pocket across a different face of the Gα subunit.

This activation model also opens the field to analyze different questions about GPCR-G protein interactions. Do receptors and G proteins that form pre-coupled complexes utilize different routes of activation? Do GPCR dimers, such as the Class C GPCRs, activate in a similar mode as the monomeric GPCRs? Do different G proteins possess multiple means of activation as an additional level of signaling selectivity? How does the helical domain return to a closed conformation upon GTP binding in order to promote complex dissociation? All of these questions will continue to challenge the field of G protein signaling, indicating that there is still much to learn about heterotrimeric G proteins and their cognate receptors. Furthermore, elucidation of each of these questions within the neural circuitry will provide researchers with more targeted and specific routes of therapeutic intervention with fewer off-target effects. Understanding GPCR-G protein interaction is paramount to understanding neuronal modulation, signal integration and aberrant circuitry.

References:


This manuscript represents the first and only crystallographic representation of the ternary complex (activated receptor bound to nucleotide-free heterotrimeric G protein). This crystal structure has become the foundation for many G protein and receptor-focused structural and dynamic studies.

enhancing or inhibiting one interaction over the other may lead to significantly less therapeutic off-target effects when dosed congruently with a modulator of that GPCR. In schizophrenia, modulators of metabotropic glutamate receptors II/III (mGluR2/3) in Phase II clinical trials have shown promise in attenuating anxiety and hallucinations yet. Yet with chronic dosing, there are some reports of negative side effects involved with prolonged disruption of the hypothalamic-pituitary-adrenal axis. As mGluR2/3 can couple to several G protein specific molecules to alleviate some of the off-target effects on steroid and hormone production. However, without a working understanding of receptor-G protein interaction and activation, knowledge-based creation and testing of such ligands are unattainable.


29. Iiri T, Farfel Z and Bourne HR (1998). G-protein diseases furnish a model for the turn-on switch. Nature. 394 (6688): 35-38. Within this manuscript, the authors proposed one of the first models of G protein activation via coupling to an activated GPCR. Within this model, known as the Lever Arm model, the beta subunit acts as lever to pry apart the G alpha domains in order to free the bound nucleotide and catalyze nucleotide exchange. This model is based primarily on structural observations.


47. Cherfils J and Chabre M (2003). Activation of G-protein Galpha subunits by receptors through Gbeta-Gamma interactions. Trends Biochem Sci. 28 (1): 13-17. This manuscript refutes the preproposed model of G protein activation known as the Lever Arm in favor of a new model known as the Gear Shift. In this paradigm, the beta subunit acts a gear shifter to bind tighter to the alpha subunit upon receptor interaction. It then shifts to allow the gamma subunit to interact with the helical domain and rearrange the conformation of the alpha subunit to create an exit tunnel for nucleotide release.


In this computational structural analysis, comparative models were utilized to envision a unified mechanism of G protein activation that incorporated EPR information, DEER distance constraints, and crystal structure contacts. This is the most “current” working model of G protein activation to date produced from the laboratories of Dr. Heidi E. Hamm and Dr. Jens Meiler.


The influence of arousal on functional connectivity dynamics

Brandon Moore

In the absence of explicit instruction or stimulation, the mammalian brain exhibits widespread spontaneous neural activity. This resting state activity is highly organized and has several emergent properties. Of particular interest are the correlations between the recorded endogenous activity in distant brain areas. Previously it has been assumed that these correlations, termed resting state functional connectivity, are fundamentally static in nature but recent evidence suggests that they vary over time. The origin of functional connectivity dynamics is currently unknown although several possible explanations have been proposed. This review summarizes the existing literature concerned with the origin of dynamic resting state neural correlations and provides evidence in favor of cortical arousal as a major factor involved in these fluctuations.

Keywords arousal, resting state, functional connectivity

Resting state functional connectivity is dynamic

One of the most striking features of the mammalian neocortex is that ongoing neural activity exists in the absence of sensory input. This endogenous activity gives rise to highly structured correlation patterns between different cortical areas. These correlations, termed functional connectivity, were first observed in neurophysiological data but have been predominantly studied using resting state functional magnetic resonance imaging (RS-fMRI) ever since the first large scale spatiotemporal functional connectivity maps were created using this method. Upon examination of these cross-cortical maps, several distinct functional resting state networks are observed. These networks are consistent with known anatomical connections although they do not necessarily reflect a causative link. This is an important distinction since increases in inter-cortical correlations can occur due to not only monosynaptic projections between two brain areas but also indirect projections or through common input (Fig. 1). Because of this property, functional connectivity studies alone are not sufficient to determine the structural nature of the brain but can be used to observe in vivo cortical association dynamics that may not be prominent or even observable in anatomical studies. Due to this exploratory nature of functional connectivity and the fact that these endogenous correlations are an evolutionary conserved property that is observed across mammalian species, resting state functional connectivity has recently become a mainstay of the inter-institutional Human Connectome Project, allowing for the study of the brain’s functional blueprint.

Due to the temporal resolution of fMRI (on the order of seconds to minutes), these studies typically employ analysis methods that assume temporal stationarity. In other words, functional connectivity is often computed over an entire imaging session and is assumed to remain static across this period of time. Although this assumption is valid with respect to known slow changes in network size and strength such as those that have been observed over childhood development, there is growing evidence for the existence of dynamics that occur much faster. Recently it has been discovered that fluctuations in cognitive load can have an impact on functionally connected networks. For example, as subjects perform n-back working memory tasks of increasing difficulty, they have an associated increase in default mode network deactivation as well as a preferential shift of connectivity from anterior to posterior regions of cingulate cortex. Learning effects have also been demonstrated to contribute to functional network dynamics. Specifically, as subjects learn a novel bimanual motor task, they exhibit changes in functional connectivity that are consistent with increased activation of the anterior cingulate cortex and a shift in connectivity from posterior to anterior regions of the brain. This suggests that functional connectivity is not static but rather reflects the dynamic nature of the brain's functional blueprint.

Keywords arousal, resting state, functional connectivity

Figure 1. Functional connectivity can reflect modular interactions from (A) monosynaptic or (B) polysynaptic projections as well as those arising from (C) a common input.
sequence, connectivity between hemispheric motor areas reduces\(^{23}\). These task-based fluctuations can occur throughout a typical scanning period (several minutes) and have been known to continue during subsequent resting state recordings\(^{24-26}\). In summary, the task engaged brain's functional network interactions are not static but rather dynamic in nature and can have effects that continue beyond the conclusion of a task.

The spatiotemporal properties of functional connectivity recorded in a task-isolated resting state are just as dynamic as the aforementioned network fluctuations\(^{33}\). Because of this observation, the RS-fMRI assumption of stationarity has been called into question\(^{34,41-44}\). Specifically, time-varying changes in both coherence and phase between the posterior cingulate cortex and other nodes in the default mode network have been observed on the order of seconds to minutes during the period of twelve minute RS-fMRI scans\(^{34}\).

Dynamic resting state correlations have also been measured with magnetoencephalography (MEG)\(^{39}\) and electrophysiology\(^{36}\), however, the source (or sources) of these fluctuations remains unknown. Unlike task-driven variations, resting state dynamics do not have an obvious origin, although several explanations have been proposed\(^{33}\). Popular explanations include noise (due to either movement or physiology), conscious brain processes, and arousal (Table 1). This brief review attempts to summarize the existing literature concerned with the source of resting state functional connectivity dynamics while discussing each of this phenomenon’s aforementioned possible origins and providing an argument for cortical arousal as a major factor in these time varying changes.

**Fluctuations in neural correlations during rest are not due to noise**

Traditionally, the seemingly random fluctuations in neural activity occurring in the absence of explicit input or output were considered to be nothing more than noise inherent to the system\(^{45}\). To an extent, this viewpoint still exists, with some in the imaging field proposing that variability of

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<td>Movement</td>
<td>Head movements during RS-fMRI studies can introduce artifacts that significantly affect correlations in the BOLD signal(^{27}).</td>
<td>In the anesthetized and head fixed monkey, where no movement can occur, functional connectivity is still dynamic(^{12}).</td>
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<td>Physiological</td>
<td>Respiratory and cardiovascular fluctuations can alter the BOLD signal introducing noise(^{28-31}).</td>
<td>Functional connectivity is minimally affected by these changes(^{26}). Fluctuations in neural correlations are also observed in MEG and LFP which are not hemodynamic(^{56,39}).</td>
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<td>Attention</td>
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<td>A subject in a resting state will often begin mind wandering(^{33,34}). Depending on what the subject is imagining, functional connectivity can be greatly affected(^{35}).</td>
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<td>Arousal</td>
<td>Significant differences in functional connectivity have been observed between sleep and waking(^{37}). Additionally, functional connectivity within the V1 columnar microcircuit has been demonstrated to vary inversely with cortical arousal(^{36}).</td>
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Table 1. A summary of the proposed explanations for resting state functional connectivity dynamics with an emphasis on the evidence in support and against each of these claims.
resting state functional connectivity could arise from MRI scanner-related artifacts, subject movement, and respiratory or cardiovascular processes. Although these factors are indeed significant sources of noise, they cannot sufficiently explain functional connectivity dynamics. For example, the majority of subject movement is mitigated in the anesthetized and head-fixed macaque, yet fluctuations in the magnitude of inter-cortical correlations in this preparation are comparable to those seen in the awake human. If a subject's movements were the cause of these fluctuations, then these dynamics should not be observable, or in the very least be severely dampened in the head-fixed anesthetized preparation, where movements are virtually eliminated.

Functional connectivity studies that utilize fMRI can be particularly susceptible to systemic noise introduced by respiratory and cardiovascular processes since they are based on a blood oxygen level dependent (BOLD) signal. Fluctuations in heart rate and arterial carbon dioxide are known to have an effect on the BOLD signal. Both respiratory and cardiovascular factors that drive changes in the BOLD signal are associated with some functional connectivity dynamics. This property, however, does not completely account for the time-varying nature of neural correlations. Resting state BOLD signals are primarily dominated by very low frequencies (<0.1 Hz), which are below the ranges for respiratory (0.1 to 0.5 Hz) and cardiac (0.6 to 1.2 Hz) noise sources. The combined contribution of these physiological noise sources has been computed and shown to be minimal (<10%), suggesting that cardiopulmonary artifacts are not the origin of resting state dynamics. Additionally, fluctuations in neural correlations have been observed in both MEG and the local field potential (LFP). Since both of these signals are of neural origin, the time variations observed in functional connectivity cannot merely be a result of any purely vascular phenomena. Although seemingly stochastic in nature, fluctuations in spontaneous neural correlations are actually highly structured and are unlikely to be a result of noise.

Fluctuations in neural correlations during rest are not due to conscious brain processes

The analysis of resting state data presents a unique challenge in that subjects are in a condition which can include a variety of conscious brain processes. For example, a RS-fMRI study participant may attend to various incidental stimuli or begin mind-wandering. These mental activities have been proposed as a possible explanation for the non-stationarity of resting state neural correlations. Notably, functional connectivity is significantly modulated during the performance of an auditory attention task. During the time it takes to perform a typical fMRI study, it is entirely plausible that a subject could shift his or her attention between a variety of different incidental stimuli such as the loud rhythmic sounds produced by an MRI scanner or any vibrations that are transferred to the scanner bed.

Without explicit stimulation, a subject may find that his or her thoughts shift across a variety of subjects over time. These shifting cognitive states can have a large effect on functional connectivity. In a recent RS-fMRI study, subjects were instructed to do nothing, recall events that happened previously that day, mentally perform subtraction, or to silently recite song lyrics. It was found that distinct functional networks were differentially strengthened dependent upon the instruction that the subject was given. Is it possible that these conscious brain processes explain the variations in functional connectivity? To approach this question, the frontoparietal resting state network was examined in anesthetized macaques. These monkeys were anesthetized using isoflurane which, when administered, results in a loss of consciousness, amnesia, analgesia, and suppression of reflexes. Animals in such a condition are not capable of the aforementioned mental behaviors, yet this study’s subjects still exhibited dynamic fluctuations in functional connectivity that were comparable to those observed in the awake human network homologue. Although conscious brain processes have been shown to cause variations in functional connectivity, they do not sufficiently account for fluctuations that are observed in the resting state.

Arousal as a potential source of resting state functional connectivity dynamics

Within the course of a day, an individual will pass through a continuum of different states of arousal, ranging from quiescent wakefulness to high vigilance. Over eighty years ago, Hans Berger observed that these shifts in behavioral arousal were associated with drastic fluctuations in recorded electrophysiological activity. These arousal-driven fluctuations range between low frequency dominated signals observed in slow-wave sleep and those comprised of higher frequencies that are indicative of wakefulness. Following this observation, it was classically held that the brain operates in a low frequency, electrophysically “synchronized” state during sleep that becomes “desynchronized” upon waking. More recent research, however, reveals an increasingly complex reality in which the level of cortical arousal continuously varies, even during periods of wakefulness rather than simply existing in the previously mentioned dichotomous states of electrophysiological synchronization. The alert and actively engaged brain is primarily dominated by high frequency cortical activity but can take on a more “synchronized” nature during drowsiness and quiescent wakefulness.
Fluctuations in cortical arousal are known to affect sensory processing across several different modalities including the auditory, somatosensory, and visual systems (for review, see Harris & Thiele\textsuperscript{57}). Shifts along the continuum of vigilance are known to toggle thalamic relay neurons between burst and tonic firing modes\textsuperscript{58,61}, alter cortical response properties\textsuperscript{62,63}, and modify the effective circuitry of inter-laminar projections by stimulating interneurons that inhibit either within or across cortical columns\textsuperscript{64}. Notably, as the measure of an awake rabbit’s electrocortical activity becomes more and more dominated by low frequencies (more “synchronized”), a condition that occurs as a subject becomes drowsy, the receptive field properties of neurons in primary visual cortex (V1) become larger\textsuperscript{65}.

Arousal fluctuations occur primarily due to a combination of subcortical factors. The bulk of these variations can be explained by increased activity in cholinergic\textsuperscript{66-69}, noradrenergic\textsuperscript{70}, and monoaminergic\textsuperscript{71-73} subcortical nuclei. These neuromodulatory projections reach the entirety of cerebral cortex\textsuperscript{74}. Given that such arousal related changes are known to occur throughout the brain, how are functionally connected networks affected? One particular neurophysiology study examined the effects of sleep and waking on the feline homologues of the default mode network and its anticorrelated network. It was discovered that negative correlations between these regions were more frequently observed during waking compared to deep sleep\textsuperscript{75}. Although these results showed an arousal related effect, it only does so for representative extremes of arousal (e.g. waking versus sleep) rather than using a continuous measure.

If there is indeed a link between variations in vigilance and functional connectivity then it would follow that graded changes in arousal should result in network fluctuations on similar time scales. To examine how a continuous measure of cortical arousal is related to resting state neural correlations, LFPs have been recorded across the depth of V1 in resting macaques as they drifted between heightened vigilance and quiescent wakefulness. Using the microcircuit of V1 as a model system, the magnitude of functional connectivity has been calculated over time and compared to a measure of arousal quantified with a novel index. It has been shown that arousal is inversely related to the strength of neural correlations in V1\textsuperscript{36}. In other words, as arousal increases, the strength of network synchrony decreases. This recent evidence, compounded with the aforementioned studies, demonstrates cortical arousal as a plausible explanation for the dynamic nature of functional connectivity.

Study of the correlations between distant brain areas is needed for the complete mapping of a functional neural “blueprint”\textsuperscript{16}. Up until recently, most resting state functional connectivity studies have assumed a level of stationarity between correlated brain areas\textsuperscript{17}. However, this assumption of stationarity has been called into question\textsuperscript{54}, and recent studies have demonstrated that resting state functional connectivity fluctuates over time in a dynamic fashion\textsuperscript{12,33,34}. Although the origins of these time-varying correlations within the resting brain are currently unknown, several possible explanations have been proposed (Table 1): the dynamics could be an effect of noise (either motion induced or physiological), conscious brain processes (like attention or mind wandering), or changes in arousal. Of the possibilities discussed in this review, cortical arousal is one of the most plausible explanations. A subject drifting between varying levels of cortical arousal will exhibit significant changes in electrocortical synchrony\textsuperscript{47,48,59}. “These fluctuations are known to affect sensory processing\textsuperscript{65,75} and are even implicated in the effective modification of the brain’s neural circuitry\textsuperscript{64}. Variations in cortical arousal occur at time scales that correspond to fluctuations in inter-cortical correlations and have recently been demonstrated to be inversely related to the magnitude of dynamic functional connectivity recorded across the V1 microcircuit\textsuperscript{36}. Since these fluctuations occur at time scales that are often beyond the temporal resolution of fMRI, further study of this phenomenon must be conducted in conjunction with other methods such as neurophysiology or MEG (for review of RS-fMRI in conjunction with other methods, see Hutchison et al.\textsuperscript{33}). Time varying changes in resting state neural correlations are associated with changes in cortical arousal; however, the extent of arousal’s contribution to functional connectivity dynamics will only be fully understood once cross-methodological approaches become more widely adopted.

Concluding Remarks

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Working Memory and its Disturbances in Schizophrenia

Monika Murphy

Schizophrenia is a severe psychiatric illness characterized by positive, negative and cognitive symptoms. The pathophysiology of schizophrenia is extremely complex, but there are certain consistent anatomical traits. These include enlarged lateral ventricles, cortical thinning and dendritic spine loss in the prefrontal cortex (PFC). Loss of dendritic spines has been repeatedly reported in postmortem studies and recapitulated in animal studies. This spine loss may be related to the working memory deficits seen in patients with schizophrenia. Working memory deficits fall into the class of cognitive symptoms of schizophrenia, which are most predictive of a patient’s outcome. Working memory and its deficiencies have been extensively researched using neuroimaging and animal studies. The PFC and the mediodorsal thalamus (MD) are two critical brain regions involved in the working memory circuit. Though much of the gross pathophysiology was studied in the context of working memory and schizophrenia, the molecular underpinnings of the deficits remain completely unknown. The following review will briefly outline the clinical features and pathophysiology of schizophrenia and discuss the components of working memory and implications in schizophrenia.

Keywords  Schizophrenia, working memory, prefrontal cortex, mediodorsal thalamus, dopamine

Introduction

Schizophrenia is a debilitating mental illness that affects nearly 1% of the population worldwide\(^1\,^2\). Clinically, schizophrenia is characterized by three major classes of symptoms: positive symptoms, negative symptoms and cognitive deficits. Positive symptoms are named as such because their presence is abnormal; these symptoms include hallucinations, delusions and disorganized speech or behavior. Conversely, negative symptoms are named as such because the absence of such features is abnormal\(^1\,^2\). In schizophrenia, negative symptoms often manifest as blunted affect, anhedonia, avolition, and paucity of speech\(^3\,^4\). Cognitive symptoms in schizophrenia include deficits in executive function, processing speed, language and working memory\(^4\). Though schizophrenia is an extremely heterogeneous disorder, cognitive symptoms are very pervasive, with nearly all patients exhibiting deficits in some capacity\(^5\,^6\). Additionally, cognitive deficits are predictive of the patient's outcome, as assessed by work outcome\(^6\).

Schizophrenia is typically treated with anti-psychotic drugs (APDs), which primarily target the positive symptoms of the illness\(^2\,^7\). There are two major classes of APDs: first-generation anti-psychotics (FGAs) and second-generation anti-psychotics (SGAs). All known APDs are antagonists of the dopamine receptor D\(_2\), though their affinities for this receptor are highly variable\(^8\,^9\). Among FGAs, affinity for the D\(_2\) receptor directly correlates with its clinical potency\(^8\,^9\). In other words, a higher affinity of the drug for the D\(_2\) receptor corresponds directly with a lower dose needed for clinically efficacy. This principle does not hold true among the SGAs, as many of the SGAs show a relatively weak affinity for the D\(_2\) receptor. The SGAs also tend to be more promiscuous, affecting many different receptors, including the serotonin receptors, 5-HT\(_{1A}\) and 5-HT\(_{2A}\)\(^10\). Despite working at varying affinities and potencies, all FGAs and SGAs antagonize the D\(_2\) receptor; this has led to the idea that dopamine plays a critical role in the pathophysiology of schizophrenia.

Pathophysiology of Schizophrenia

A number of anatomical changes have been found in patients who have received a diagnosis of schizophrenia. Enlargement of the lateral ventricles is a consistent finding in both living and postmortem brains of schizophrenia patients\(^11\,^12\). One study, using computer tomography (CT), showed that over half of the patients in the study with schizophrenia displayed a ventricular brain ratio (VBR) that was over two standard deviations above the mean VBR of the control group\(^11\). Schizophrenic brains were reported to weigh approximately 6% less than control brains; this could not be attributed to size of the person (as measured by body weight and height)\(^13\). The enlargement of ventricles as measured by the VBR, along with no change in head size\(^14\) and loss of brain mass suggests a loss of brain tissue.
In a study to determine changes in cortical neuron number, Pakkenberg used an unbiased stereology approach to count neurons in the neocortex of schizophrenic and control post mortem brains. In this study, there was no significant difference in the number of neurons, indicating that change in brain weight is not due simply to a loss of neurons.

Similar to the increase in VBR ratio, Selem et al. found a decrease in cortical thickness in Brodmann’s area 9 (BA9) and Brodmann’s area 46 (BA46) in post-mortem brains of patients with schizophrenia, as compared to controls. Both BA9 and BA46 correspond to the dorsolateral prefrontal cortex (DLPFC), a region critical to working memory function, which will subsequently be discussed in detail. As described previously, cortical thinning seen in this study is not caused by a loss of neurons. Instead, neuronal density is significantly increased in both BA9 and BA46 in the post-mortem brains of patients diagnosed with schizophrenia. Additionally, only a slight decrease in neuronal size was shown and this decrease was only seen in layer 3 of BA 9. No significant decrease in neuronal size was seen when all layers of cortex were grouped together.

Current evidence in schizophrenia literature indicates there is a loss of brain mass and an increase in neuron density, but no change in cell number. This information led Goldman-Rakic and Selem to develop the reduced neuropil hypothesis of schizophrenia. Neuropol consists of dendrites, axons, dendritic spines and axon terminals, but does not include cell bodies. The reduced neuropil hypothesis, therefore, posits that cortical neurons atrophy in schizophrenia and lose neuropol, which can lead to tighter packing of the neurons and cortical thinning, both of which have been documented in the literature and outlined above.

A lower density of dendritic spines was reported in the cortex of post mortem schizophrenic brains, lending further evidence for the reduced neuropil hypothesis. Glantz and Lewis specifically analyzed dendritic spine density in layer 3 of BA46, considered part of the DLPFC. Spine analysis was performed using the golgi technique. They reported a 23% decrease in spine density on deep layer 3 pyramidal neurons in schizophrenic brains, as compared to normal controls, and a similar decrease when compared to non-schizophrenic psychiatric subjects. This study also reported no significant difference between non-schizophrenic psychiatric controls, some of whom had taken APDs, and normal controls in spine density, indicating that treatment with antipsychotics was unlikely to cause the drastic decrease in spine density seen in schizophrenic brains. Interestingly, the deep portion of layer 3 of the DLPFC receives the densest dopamine innervation and is a major source of projections to the mediodorsal nucleus of the thalamus (MD), implicating it in working memory.

Studies of spine density have also been performed in rodents. Wang and Deutch showed that dopamine de-\nervation of the prefrontal cortex (PFC) of rats induced a significant decrease in spine density when compared to sham surgery controls. Dopamine depletion of the PFC was previously shown to cause significant working memory deficits. In this study, the prelimbic area of the PFC was analyzed because it most resembles the DLPFC of primates based on hodology and is thought to subserve working memory in the rodent. Additionally, this study focused on layer 5 of the prelimbic PFC because, in rodents, it receives a dense dopamine innervation. The decrease in spine density was not seen in motor cortex where there is no dopamine innervation. This supports the notion that dopamine innervation has an effect on spines and when it is removed, spines are not properly maintained.

Components of Working Memory

Cognitive deficits are a pervasive feature of schizophrenia. The severity of the cognitive deficits in patients is predictive of social functioning, including the ability to return to work, and this relationship is stable over time. It is important to note that severity of positive symptoms does not correlate with social functioning, despite the fact that all APDs target these symptoms. One of the most studied cognitive deficits in schizophrenia is impaired working memory. In brief, working memory temporarily maintains and manipulates information that is needed to achieve a goal or complete a task. A typical example of working memory is remembering a phone number for a short period of time when you are not able to write it down immediately. Working memory impairments are seen in most patients with schizophrenia and are also seen in first-degree relatives of schizophrenics and other people at high risk for psychosis. These impairments were also seen in first episode patients, the majority of whom were naïve to antipsychotic medication, indicating that the cognitive impairment found in schizophrenia is likely due to an underlying pathology and not due to treatment with APDs, chronic illness or extended institutionalization. All of the data presented thus far indicate that the circuits underlying working memory play a key role in the pathology of schizophrenia and may help elucidate its etiology.

Two major brain areas are implicated in working memory: the PFC and the MD. In both primates and rodents, the PFC and MD have reciprocal glutamatergic connections. In primates, these connections are primarily between the MD and the DLPFC, defined as Brodmann’s areas 9, 10, and 46. In rodents, however, the connections are between the MD and the medial PFC (mPFC), specifi-
ly the prelimbic area (PrL) of the mPFC. As mentioned previously, the DLPFC and mPFC are topographically different, but have similar hodology and subserve comparable functions in primates and rodents\(^\text{43}\).

In order to determine that the PFC and MD were critical to working memory function, a number of studies were performed in which these areas were lesioned and animals were tested with behavioral paradigms relevant to working memory. For example, Isseroff et al. showed that lesioning the MD of young adult rhesus monkeys caused significant impairments in performance on a delayed alternation task and a delayed response memory task\(^\text{44}\). In this study, however, there were no impairments in pattern discrimination or associative memory tasks, indicating that the impairment caused by an MD lesion is limited to the working memory system. Another group showed that MD lesions in nonhuman primates caused impairment in delayed nonmatching to sample tasks, but again left pattern recognition and associative memory intact\(^\text{45,46}\). When lesions were inflicted in the DLPFC of nonhuman primates, the impairments in the tasks mentioned above were identical to those seen in the MD lesions, giving further support to the idea that the MD and the DLPFC form a circuit critically involved in working memory function\(^\text{47,48}\).

Similar studies were performed in rodents. Beracochea et al. investigated the role of the MD in rats by performing a neurotoxin lesion of the MD and testing the rats using behavioral tasks\(^\text{49}\). MD-lesioned rats showed no differences in behavior on spatial paradigms such as the radial arm maze. Once a time delay was introduced into a task, as in the delayed alternation maze, the MD-lesioned rats showed significant impairments. The MD-lesioned rats took many more trials to learn the task and also performed poorly compared to both sham-lesioned and anterior thalamus-lesioned rats. A delayed nonmatch-to-sample paradigm revealed comparable impairments in MD-lesioned rats\(^\text{50}\). Very similar results were seen when the mPFC was lesioned in rats tested with the same behavioral paradigms\(^\text{51,52}\). Together, these data suggest that both the MD and PFC are critical areas of the brain for proper working memory function.

Until recently, efforts to detail the working memory circuitry in the rodent closely paralleled work done in nonhuman primates. With the advent of new technologies such as optogenetics and Designer Receptors Exclusively Activated by Designer Drugs (DREADD), it is becoming increasingly possible to perform fine manipulations on the circuits involved in working memory in order to truly understand the way they work in a living animal. These technologies are new, but have already been implemented in a number of rodent studies that aim to dissect different parts of the working memory circuit.

One group attempted to bridge the gap between traditional lesion studies and optogenetics by utilizing a paradigm in which three sets of mice underwent inactivation of the mPFC by three different methods\(^\text{52}\). One set of mice underwent an excitotoxic lesion of the mPFC, a second set of mice underwent a local inactivation of the mPFC by muscimol and a third set of mice expressed channelrhodopsin-2 in parvalbumin-expressing interneurons, which could be stimulated by light to release GABA, inactivating local pyramidal cells. In this experiment, all three sets of animals were tested using an operant delayed alternation task and all three of the experimental groups showed impaired performance on the behavioral test, even if they had been pre-trained on the task. This indicates that the mPFC is not only critical for learning a task, but is also important for successful performance of the task. Additionally, this study confirms that inactivation of pyramidal cells within a brain region by different methods can produce the same behavioral outcome.

To determine the role of the MD in working memory, Parnaudeau et al. used the DREADD system to induce MD hypofunction in mice and then analyzed the performance of the mice on different behavioral paradigms\(^\text{53,54}\). The DREADD system allows a temporary and reversible inactivation of a brain region, whereas a lesion is permanent. Additionally, any behavioral changes seen in a lesion study may not be due to the lesion itself, but instead due to the manner in which the brain compensates for the damage\(^\text{42}\). The use and effect of clozapine-N-oxide (CNO), the designer drug of the DREADD system, is temporary and it is unlikely to induce permanent changes in the brain. Using DREADD in the MD, Parnaudeau et al. were able to show that induced MD hypofunction caused impairments in working memory\(^\text{54}\). This study measured working memory by looking at tasks that specifically address goal-directed behavior and cognitive inflexibility. Using the DREADD technology, Parnaudeau et al. were able to more precisely analyze the working memory circuit.

Another group aimed to dissect the time course of a working memory task through an optogenetic approach by silencing the neurons using light stimulation of Archaerhodopsin-T receptor at different points in the behavioral task\(^\text{55}\). This group chose to look at trace fear conditioning, in which working memory is needed to bind together noncontiguous stimuli. When the prelimbic area of the PFC was silenced in the period of time between the presentation of a cue and the foot shock, the rats showed less freezing behavior, indicating that the cue and foot shock were not properly associated. This impairment in association was not observed in the association of the chamber to the shock, nor when the PrL area was silenced during only the cue phase, indicating that the maintenance of information and the association of the two stimuli requires activity of the PrL area of the PFC. Though this
study of working memory is related to the amygdala mPFC circuit, it offers good evidence that the mPFC is critical for maintenance of information in working memory. It also provides an experimental paradigm that can be altered and used to test various components of working memory.

Working Memory Disruptions in Schizophrenia

There is no true animal model of schizophrenia. To study the specific working memory deficits of schizophrenia, therefore, it is necessary to perform research on humans. Typically, working memory in schizophrenia is studied with neuroimaging techniques, such as positron electron tomography (PET) and functional magnetic resonance imaging (fMRI). One meta-analysis of 60 fMRI and PET working memory studies found activation of the DLPFC in working memory tasks that required updating of information in working memory or memory for the sequence of stimuli presented. The results of this meta-analysis showed that working memory representations are organized by process (i.e., updating of information, manipulation of information, temporal sequence) in the brain, as opposed to by the type of material presented (i.e., spatial memory, object memory). This pattern corresponds well with the fact that most patients with schizophrenia show deficits in specific working memory processes across different material types. Additionally, schizophrenic patients exhibit abnormal functional connectivity during working memory tasks. A decrease in fronto-parietal coupling correlated well with poorer performance on a working memory task, indicating that this connectivity may be directly related to the working memory impairments seen in schizophrenia.

Conclusion

This review highlighted some of the key features of the pathophysiology and cognitive deficits seen in schizophrenia. However, little is known about the molecules that underlie the etiology and pathology. Despite numerous anatomical, circuit-level, and behavioral studies of working memory deficits in schizophrenia, the molecules subserving this defined circuit remain elusive. More molecular studies are needed to address this question. Dopamine depletion of the prefrontal cortex in rats leads to working memory deficits. Unpublished data from our lab shows that there is selective loss of dendritic spines on layer 5 pyramidal cells of the PrL area of the PFC that project to the MD in response to dopamine depletion. Molecular differences between MD-projecting PFC pyramidal cells and pyramidal cells that project to other targets are unknown, despite the fact that they seem to underlie working memory deficits.

References


Figure 1. Schematic illustration of PFC innervation for working memory and schizophrenia. This limited schematic shows the inputs and outputs of the PFC. The MD has reciprocal glutamatergic projections with the PFC. The VTA provides the dopaminergic innervation of the PFC, with the densest innervation in layer 5. Many other brain areas have reciprocal projections with the PFC as well. Though the circuits have been characterized, molecular differences between cells that project to different targets are completely unknown. This is important because pyramidal cells that project to the MD from the PFC are implicated in working memory function.


This paper was the first to report loss of dendritic spines in the DLPFC in post mortem brains of patients diagnosed with schizophrenia, giving evidence for the reduced neuropil hypothesis.


Performed in our lab, this paper shows dendritic spine loss in rodents in response to a dopamine depletion of the PFC. The loss of dendritic spines was reversed when rats were treated with an atypical APD, but not when treated with a typical APD.


This paper showed that working memory in the PFC of non-human primates is significantly impaired when dopamine signaling through D1 receptors is inhibited using antagonists.


Using three different mPFC inactivation techniques, this group showed that inactivation of the PFC leads to working memory deficits.


Model Systems for Studying GGE-associated GABA<sub>A</sub> Receptor SubunitMutations

Dingding Shen

Mutations in GABA<sub>A</sub> receptor subunit genes (GABRA1, GABRB3, GABRG2 and GABRD) have been associated with genetic generalized epilepsies (GGEs). The functional consequences of these GGE-associated GABA<sub>A</sub> receptor subunit mutations have been studied in different experimental models, providing significant insights in GGE epileptogenesis. It is now clear that these mutations alter GABA<sub>A</sub> receptor function and/or impair receptor biogenesis, and thereby result in disinhibition and increase seizure susceptibility. In this review, we will discuss different model systems as well as their valuable contributions to our understanding of GGE-associated GABA<sub>A</sub> receptor subunit mutations, highlighting their advantages and limitations.

**Keywords** Genetic generalized epilepsy, GABA<sub>A</sub> receptor mutation, heterologous cells, cultured neurons, mouse model

**Introduction**

Genetic generalized epilepsies<sup>a</sup> (GGEs) are common and represent approximately 30% of all epilepsies<sup>1, 2</sup>. As defined in the current classification of the International League Against Epilepsy (ILAE)<sup>3</sup>, GGEs include multiple epilepsy syndromes, varying in clinical severity from relatively benign childhood absence epilepsy (CAE) to the catastrophic epileptic encephalopathy Dravet syndrome. Numerous mutations of voltage- and ligand-gated ion channel genes have been associated with GGEs, leading to the concept that GGEs are a family of channelopathies. These ion channels include voltage-gated sodium channels<sup>4, 5</sup>, voltage-gated potassium channels<sup>6</sup>, voltage-gated calcium channels<sup>7</sup> as well as GABA<sub>A</sub> receptors<sup>8, 9</sup>.

Although genetic studies combined with careful clinical evaluations of epilepsy phenotypes are crucial for the identification of GGE-associated GABA<sub>A</sub> receptor mutations, these studies cannot provide a complete clarification of genotype-phenotype relationships. Therefore, it is usually necessary to perform functional studies in vitro and in vivo in order to completely disclose the pathophysiological effects of GABA<sub>A</sub> receptor subunit mutations. In general, in vitro studies permit direct investigation on the effects of the mutation on GABA<sub>A</sub> receptor biogenesis and function in a relatively simple system without the impact of complex neuronal development or network activities. They allow a relatively fast screening of mutants and thus are usually the methods of first choice, but they do not fully recapitulate the situations in vivo. Compared with them, genetically modified knock-in mice have the advantage of allowing evaluation of the impact of the mutant GABA<sub>A</sub> receptors in the complex context of an intact living organism. It is important that results of functional studies should be interpreted carefully considering the characteristics of the experimental systems used<sup>10</sup>.

We will outline here the model systems that are employed for the functional analysis of GGE-associated GABA<sub>A</sub> receptor subunit mutations, focusing on their pros and cons. First, we will talk about GABA<sub>A</sub> receptors and the mutations associated with GGEs. Next, several in vitro model systems including heterologous cells and cultured neurons will be addressed. Finally, we will review studies of a knock-in mouse model.

**GGEs and GABA<sub>A</sub> receptor mutations**

GABA<sub>A</sub> receptors are the primary mediators of fast inhibitory synaptic transmission in the central nervous system. Like other members of the Cys-loop superfamily of ligand-gated ion channels, they are heteropentamers assembled from multiple homologous subunit subtypes (α1–α6, β1–β3, γ1–γ3, δ, ε, π, θ, and ρ1–ρ3)<sup>11</sup>. All subunits contain a large extracellular N-terminal domain, four transmembrane domains (M1-M4) and a small C-terminal domain. Most GABA<sub>A</sub> receptors are thought to comprise two α subunits, two β subunits, and one γ or δ subunit<sup>12-14</sup>. GABA<sub>A</sub> receptors regulate moment-to-moment brain function by mediating both phasic and tonic inhibitory synaptic transmission. During brain development, GABA<sub>A</sub> receptors also play a critical role in regulating neuronal proliferation, migration, differentiation and synaptogenesis<sup>15</sup>. Moreover, enhancement of GABA<sub>A</sub> receptor function is the basis of action for a number of antiepileptic drugs.

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<sup>a.</sup> **Genetic generalized epilepsies:** generalized epilepsies due to single gene disorders or multigenic disorders with complex inheritance, and in which there is no gross neuroanatomical or pathologic abnormality.
of antiepileptic drugs including benzodiazepines and barbiturates.

Therefore, it is not surprising that mutations in GABA$_A$ receptor subunit human epilepsy (HPE) genes GABRA1, GABRB3, and GABRG2, and variants in GABRD are involved in multiple GGE syndromes. These include nonsense, missense, insertion or deletion/frameshift mutations in translated regions as well as promoter and splice donor site mutations in untranslated regions.

Model systems for functional analysis of GGE-associated GABA$_A$ receptor subunit mutations

In Vitro Models

In vitro models can be divided into two groups: heterologous cells (including *Xenopus* oocytes and mammalian cell lines) and cultured neurons. The pathophysiological effects of GABA$_A$ receptor subunit mutations have been primarily evaluated thus far using different in vitro models.

Heterologous cells

Heterologous cells are non-neuronal cells that have small endogenous currents. They are relatively easy to use and thus are considered as the necessary first step to study GABA$_A$ receptor subunit mutations.

*Xenopus* oocytes

The oocytes of the clawed African frog *Xenopus* laevis have been widely used for many years as a heterologous expression system for studying ion channels because they provide several advantages. These huge cells (1mm-1.3mm in diameter) with few endogenous channels are cheap and easy to handle. Furthermore, they can efficiently translate exogenous mRNAs and allow subsequent long and stable electrophysiological recordings of ion channel currents. Initial functional characterizations of several GABA$_A$ receptor mutations were first studied by using this expression system. For example, expression of the GABRG2(K328M) mutation in oocytes resulted in a decrease in the amplitude of GABA activated currents. In contrast, the GABRG2(R82Q) mutation didn’t alter GABA-mediated currents but caused a loss of current enhancement by benzodiazepine.

However, use of the *Xenopus* oocytes expression system requires consideration of several limitations; the most serious is its non-mammalian cell background. It has been shown that GABA$_A$ receptor assembly in *Xenopus* oocytes is more promiscuous than in mammalian cells. Furthermore, the oocyte system has relatively slow temporal resolution, which makes it difficult to explore the rapid kinetic properties most relevant to the time scale of synaptic transmission, such as activation, desensitization, and deactivation. Therefore, *Xenopus* oocytes are now used less often for studies of GABA$_A$ receptor mutations.

Cultured Mammalian Cell Lines

Mammalian cell lines have now become the current standard for studying GABA$_A$ receptor mutations. The most frequently used cells are human embryonic kidney (HEK) cells. The principle characteristics that have made the HEK cell a robust and reliable platform in which to express mutant GABA$_A$ receptors are: easy maintenance and quick growth, high efficiency of transfection and protein production using inexpensive methods, small endogenous currents, and small cell size with minimal processes appropriate for voltage-clamp recordings. Other popular cell lines include COS7 cells, HeLa cells, etc. The evaluation of GABA$_A$ receptor mutations based on the combination of traditional electrophysiological methods and biochemical methods by overexpressing them in heterologous mammalian cell lines has provided substantial knowledge on the cellular and molecular mechanisms underlying GGE epileptogenesis. These mechanisms include nonsense-mediated mRNA decay (NMD), ER retention with impaired oligomerization, ER-associated degradation (ERAD), and gating defects. In an attempt to give significant examples of the value of HEK cells in studying GGE-associated GABA$_A$ receptor mutations, we will focus on a nonconserved missense mutation, GABRA1(A322D), which causes autosomal dominant juvenile myoclonic epilepsy (ADJME) and has been extensively studied in vitro. Electrophysiological experiments revealed altered function of recombinant GABA$_A$ receptors containing the A322D mutation. For instance, by using an ultrafast application system, mutant receptors showed decreased macroscopic current amplitudes at saturating GABA concentrations, a significantly reduced affinity to GABA, and accelerated deactivation compared to the wild type. In addition, western blot and biotinylation assays revealed that the A322D mutation resulted in decreased $\alpha_1$ total and surface expression. To investigate the mechanism involved in this reduction, many other biochemical experiments have been performed, demonstrating that A322D mutation decreased the half-life of $\alpha_1$ subunits and loss of the misfolded $\alpha_1$(A322D) subunits resulted from rapid endoplasmic reticulum-associated degradation (ERAD) via the ubiquitination pathway.

**Benzodiazepines**: Compounds that potentiate the response elicited by GABA by binding at the interface between $\alpha$ and $\gamma$ subunits of GABA$_A$ receptors; pharmacologically active with anticonvulsant, anxiolytic, amnesic and sedative effects.
uitin-proteasome system and lysosome-autophagy pathway. Furthermore, the A322D mutation was shown to confer a dominant negative effect that may contribute to the epilepsy phenotype based on evidence from flow cytometry and fluorescent resonance energy transfer (FRET) experiments.

The results from these studies are relevant to effects of the mutations in vivo because fundamental features of subunit translation, folding and oligomerization, and receptor assembly and trafficking are highly conserved between heterologous cells and neurons. However, GABA<sub>α</sub> receptor expression and function in heterologous cells and in vivo could differ. Heterologous cells do not provide a neuronal background, in that they don’t have endogenous GABA<sub>α</sub> receptor subunits and also lack many neuron-specific GABA<sub>α</sub> receptor-associated proteins that play important roles in GABA<sub>α</sub> receptor biogenesis, trafficking and cell surface clustering and stabilization. In addition, it is impossible to study the effect of mutations on GABAergic synaptic transmission in heterologous cells because they do not form synapses. Moreover, mutations may alter the subcellular distribution of GABA<sub>α</sub> receptors, but this effect cannot be observed in non-polarized heterologous cells.

Additionally, it should also be noted that functional studies in heterologous expression systems have provided inconsistent and ambiguous results. For example, regarding whether there is altered diazepam sensitivity associated with GABRG2(Q390X) mutation, some revealed altered benzodiazepine binding, while other groups reported intact binding between benzodiazepine and mutant GABA<sub>α</sub> receptors. This discrepancy may arise from differences in transfection methods or culturing conditions that may alter the functional consequences of mutant GABA<sub>α</sub> receptor subunits.

**Cultured Neurons**

Cultured neurons can overcome most, if not all of the limitations of heterologous cells mentioned above. In addition to expressing neuronal specific proteins, their polarity and subcellular specialization allow for investigation on the targeting of mutant GABA<sub>α</sub> receptors to synaptic, perisynaptic or extrasynaptic sites. Moreover, they can form active GABAergic synapses and functional neuronal networks which obviate the need for exogenous applications of GABA.

Several GGE-associated GABA<sub>α</sub> receptor subunit mutations including GABRG2(R82Q), GABRG2(K328M), GABRG2(Q390X) and GABRA1(A322D) have been studied in cultured neurons. Unsurprisingly, these studies confirmed some of the initial observations obtained in the heterologous cells. For example, studies in HEK cells revealed that mutant γ2(Q390X) subunit was immature and retained in the ER, resulting in loss of function of the γ2 subunit. In addition, γ2(Q390X) subunit exhibited a dominant negative effect by impairing assembly and trafficking of wild type partnering subunits. Similarly, when coexpressed with α1 and β2 subunits, γ2(Q390X) subunits were haploinsufficient with minimal expression on the surface of hippocampal neurons. Electrophysiological experiments in neurons demonstrated that heterozygous α1β2γ2(Q390X) receptor current amplitudes were less than half of wild type peak current amplitudes, confirming the dominant negative effect of mutant γ2(Q390X) subunits.

Studies in cultured neurons also revealed partially different or completely new findings that could not have been predicted from the heterologous expression system alone. For instance, expression of the γ2(R82Q) subunit in hippocampal neurons selectively reduced extrasynaptic tonic GABAergic currents but had no effect on synaptic phasic inhibition, whereas in transfected HEK cells only a general deficit in GABAergic signaling was detected. In addition, observations made in hippocampal neurons revealed that the GABRG2(R82Q) mutation reduced α5 surface expression. This finding would not have been observed in heterologous cell lines that do not express either endogenous GABA<sub>α</sub> receptor subunits or neuron-specific proteins such as radixin, which associates with the α5 subunit.

Transfected neurons in primary cultures can be a better experimental model in comparison with heterologous cells and a step forward in studying physiological effects of mutant GABA<sub>α</sub> receptor subunits. However, it must be highlighted that they still do not complete reproduce in vivo conditions because with transfection, the exogenous GABA<sub>α</sub> receptors are overexpressed at non-physiological levels.

**Genetically modified mice**

Epilepsy is a complex disease of neuronal networks involving the interaction of many cell types in different brain regions that can be influenced by GABA<sub>α</sub> receptor mutations. Although there is no doubt that in vitro studies have and will continue to shed light on the cellular and molecular effects of various GABA<sub>α</sub> receptor subunit mutations on GABAergic physiology, building a complete understanding of epileptogenesis in GGEs requires models that enable investigation at different organization levels and temporal scales. Genetically modified knock-in mice harboring homologous human GABA<sub>α</sub> receptor mutations preserve the complexity of the nervous system and better recapitulate real physiopathological conditions. Therefore, they are one of the best available systems to investigate mechanisms underlying GGEs.
Currently, the availability of knock-in mouse models that mimic human GGEs arising from GABA_\text{A} receptor subunit mutations is extremely poor because they are always costly and time-consuming to generate. To date, only the GABRG2(R82Q) mutation knock-in mice have been made and studied\textsuperscript{66-70}. I will summarize here the phenotype characteristics of this mouse model and evaluate its contributions to a complete understanding of epileptogenesis in GGE.

Heterozygous mice harboring the GABRG2(R82Q) mutation recapitulate the two major seizure types seen in human patients, including childhood absence epilepsy (CAE) and febrile seizures (FS). Consistent with the findings in heterologous cells and in cultured neurons, the mutation substantially reduced \gamma 2 subunit surface expression in the mouse brain\textsuperscript{66}. However, in contrast to the dominant negative effect of GABRG2(R82Q) mutation observed in cell-culture experiments, no change of \alpha 1 subunit expression was detected in embryonic neuron cultures from GABRG2 (R82Q) knock-in mice\textsuperscript{66}. This finding highlights a need for caution when using in vitro findings to explain the pathophysiological mechanisms of GABA_\text{A} receptor subunit mutations.

Moreover, investigations in this mouse model also revealed some important findings that could not be predicted in vitro. Analysis of synaptic inhibition demonstrated significantly reduced miniature IPSC amplitudes in the somatosensory cortex (layer 2/3 pyramidal cortical neurons), with no change in the thalamic reticular nucleus or ventrobasal thalamus\textsuperscript{66}. This generated the hypothesis that a reduction in cortical GABA_\text{A} receptor-mediated inhibition may underlie CAE epileptogenesis. Furthermore, it was demonstrated that GABRG2(R82Q) mutation causes CAE and FS through distinct molecular mechanisms, and these two phenotypes have different sensitivity to genetic background\textsuperscript{70}.

To investigate the developmental impact of the GABRG2 (R82Q) mutation, a tetracycline-based conditional GABRG2(R82Q) knock-in model that enabled a forebrain-specific activation of the R82Q allele at specific times during development was created\textsuperscript{67}. Seizure susceptibility was significantly reduced in mice where the R82Q allele was suppressed during development, suggesting that GABRG2 (R82Q) mutation impacts network stability during a critical developmental period.

However, one limitation of the knock-in mouse model is that the functional effects of the mutation are not studied in humans. The genetic background that may influence GABA_\text{A} receptor modulation, interactions and functions is different between mice and humans. Recently, a promising technique that uses neurons differentiated from induced pluripotent stem cells (iPSCs) generated from the skin fibroblasts of Dravet syndrome patients have allowed the study of sodium channel mutations in human neurons maintaining the patient’s genetic background\textsuperscript{71,72}. The human iPSCs may provide a remarkable platform for understanding GGE-associated GABA_\text{A} receptor mutations in the future.

Despite the limitation mentioned above, the GABRG2 (R82Q) mouse has provided important insights into how a human GABA_\text{A} receptor subunit mutation can impact GABAergic inhibition in small time scales, and impact neurodevelopment and consequently increases seizure susceptibility in longer time scales\textsuperscript{73}. Future work with genetically modified mice carrying other GABA_\text{A} receptor subunit mutations will better enable us to determine the functional effect of these mutations.

Concluding remarks

Functional studies based on different in vitro models have characterized the molecular effects of various GGE-associated GABA_\text{A} receptor subunit mutations in great detail. However, there is still lack of understanding of the impact of these mutations in complex neuronal context during development due to the limited availability of knock-in mouse models. Further studies require the critical evaluation and integration of the results obtained using different experimental systems that together span genetic, molecular, cellular, synaptic, network and whole organism levels. Exploiting the specific advantages and keeping in mind all the limitations of different model systems will help us better understand how these GABA_\text{A} receptor subunit gene mutations cause GGE, and hopefully facilitate development of therapeutic approaches in the future.

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Filling in the gap: Ceftriaxone mediated transcriptional control of glutamate transporters in astrocytes

Chelsea Snarrenberg

Glutamate (GLU) is the major excitatory neurotransmitter in the central nervous system, and perturbations in GLU signaling are linked to many diseases including amyotrophic lateral sclerosis (ALS), addiction, and ischemia. Identifying drugs currently approved as treatments for other illnesses by the Food and Drug Administration (FDA) as therapeutics to restore GLU homeostasis is a high priority for the field. Ceftriaxone, a β-lactam antibiotic, restores GLU homeostasis in disease states by up-regulating expression of the primary astrocytic GLU transporter, GLT1/EAAT2, and the cystine/GLU exchanger, xCT. The molecular mechanism by which ceftriaxone induces transcription of these genes in order to offer neuroprotection remains unknown. This review will describe transcriptional regulation of GLT and xCT as a means to reveal potential pathways in which ceftriaxone interacts to regulate gene expression and provide insight into areas that require further research.

Keywords  Glutamate, ceftriaxone, GLT1, xCT

Introduction

Neurological and psychiatric disorders are broad terms that encompass many diseases of the nervous system. The treatments for these disorders are still relatively indirect and target symptom alleviation as opposed to directly treating the molecular mechanism at the crux of these disorders. In order to expedite treatments available for brain disorders, researchers are taking a targeted approach of screening FDA approved drugs that may be efficacious as treatments or point to a mechanistic drug target for the disorder of interest.

Dysfunction in GLU clearance and GLU transporters is implicated in disorders such as stroke, schizophrenia, addiction, ALS and others. Rothstein et al. sought to identify drugs that increased GLU transporter expression. β-lactam antibiotics were identified in this screen as class of FDA approved drugs that increase expression of the astrocytic GLU transporter 1 (GLT1), the primary transporter responsible for clearance of synaptic GLU. This study specifically characterized ceftriaxone, a cephalosporin β-lactam antibiotic used to treat bacterial meningitis, as a potential therapeutic for disorders linked to disrupted GLU homeostasis.

Research regarding ceftriaxone’s effects in the CNS expanded immensely in the years following Rothstein’s discovery. Currently, ceftriaxone is thought to be neuroprotective in astrocytes by 1) preventing GLU provoked excitotoxicity by up-regulation of GLT1 and 2) by up-regulating system xCT, specifically the GLU/cystine antiporter component, xCT, to protect against oxidative stress. The goal of this review is to discuss the known mechanisms and pathways that regulate transcription of these astrocytic transporters. By understanding endogenous gene expression regulation, we seek to identify areas that require further research and avenues in which ceftriaxone may affect transcriptional regulation.

Transcriptional regulation of the primary astrocyte excitatory amino acid transporter, GLT1

Ceftriaxone’s induction of GLT1 expression as shown by Rothstein et al. suggests that targeting the mechanism of expression change may offer neuroprotection in a wide array of neurological diseases. In order to understand how a β-lactam antibiotic leads to increased GLT1 expression, researchers must understand the endogenous regulation of GLT1 transcription in astrocytes. Numerous exogenous factors increase GLT1 expression but this review will focus on transcriptional modulation by nuclear factor-kB (NFkB) in response to various administered agents such as dibutyryl-cAMP (dbcAMP), epidermal growth factor (EGF), tumor necrosis factor alpha (TNFα), etc (Figure1a).

cAMP analog induction of GLT1 transcription

cAMP is a powerful second messenger transducer of many signaling cascades. Typically, ligand activation of excitatory G-protein coupled receptors (GPCRs) leads to G-protein activation of adenyl cyclase to increase intracel-
From here, cAMP initiates a host of signaling cascades, such as activation of protein kinase A (PKA). Treatment of primary astrocyte cultures with cAMP membrane permeable analogs, such as dbcAMP, results in dramatic morphological changes, astrocytic differentiation, and an increase in GLT1 expression. Work by Zelenaia et al. suggests that dbcAMP signals through cAMP dependent protein kinase A (PKA) upstream of phosphoinositide 3-kinase (PI3K) to permit NFκB mediated increase of GLT1 expression in primary rat astrocyte cultures.

**EGF induction of GLT1 transcription**

The Zelenaia study also demonstrated that EGF treatment induces GLT1 protein expression in a pathway dependent on the transcription factor NFκB in primary astrocyte cell culture. EGF initiates a signaling cascade via EGF receptor (EGFR) dimerization and subsequent auto-phosphorylation of intracellular tyrosine residues of these receptors. Activation of EGFR via EGF coordinates PI3K activation and downstream signaling resulting in the activation of NFκB thereby controlling GLT1 expression. Two putative NFκB binding sites, -583 and -251 in the 5’ flanking region of the GLT1 promoter are required for EGF-activated expression. Furthermore, the EGF signaling cascade works in a non-canonical way. NFκB constitutively binds to the GLT1 promoter and can induce or repress transcription depending on the sites of occupation in the promoter region and the presence of other bound transcription factors. Sitcheran et al. suggests that EGF treatment promotes the induction of GLT1 transcription by activating the constitutively bound NFκB.

**TNFa repression of GLT1 transcription**

In contrast to the induction of expression by EGF through NFκB, the cytokine TNFa is reported to repress GLT1 gene expression through binding of NFκB at the -583 and +263 putative consensus sites in the 5’ region of the GLT1 promoter. TNFa transcriptional repression of GLT1 by NFκB must overcome the constitutive, positive regulation of GLT1 by NFκB. Shown to actively repress GLT1 expression, TNFa signals through the canonical pathway of inhibitor κB (IKB) phosphorylation by the kinase IKKB. Proteosomal degradation of phosphorylated and ubiquitinated IκB allows unimpeded NFκB to translocate to the nucleus in order to bind the GLT1 promoter. Additionally, evidence supports that TNFa also results in N-myc, another transcription factor, recruitment to the GLT1 promoter to convey further transcriptional repression by possibly occluding NFκB activation of the promoter. As a whole, this negative regulation further supports that specific NFκB sites may preferentially activate or repress expression of GLT1. Thus, having multiple NFκB sites allows finely tuned regulation of GLT1 transcription, and suggests that these sites could be preferentially targeted through pharmacological intervention in order to provide precise control of gene expression allowing varying levels of neuroprotection.

**Neuronal induction of GLT1 transcription**

Perhaps the most functionally and physiologically relevant modulator of GLT1 expression regulation in astrocytes is the presence of neurons. GLT1 is only expressed at low levels in primary astrocyte cultures; however, when co-cultured with neurons astrocytes differentiate and demonstrate greatly increased expression of GLT1. While neuronal dependent induction of GLT1 in astrocytes is an accepted phenomenon, only recently have researchers begun to uncover the mechanism by which this occurs. One study provides evidence that neuronal presynaptic terminals regulate astrocytic protein expression, including GLT1, by secreted factors from the neuron and through direct neuron to astrocyte membrane contact. Furthermore, this study implies a mechanism for transcriptional activation by demonstrating that an NFκB binding partner, kappa B-motif binding phosphoprotein (KBBP), is recruited to the promoter of GLT1 for neuron-dependent transcriptional activation in vivo.

Ghosh et al. demonstrated NFκB’s involvement in neuronal dependent increase in primary astrocyte GLT1 expression. Neuronal induction of GLT1 is propagated through NFκB binding to the putative consensus sequences -583 and -251 in the 5’ flanking region of the promoter, yet the -272 NFκB binding site does not seem to be required for this mechanism of regulation. This pattern of NFκB binding is similar to that induced by EGF treatment. Neuronal culture media (NCM) caused an up-regulation of GLT1 expression in primary astrocyte cultures through a non-EGFR, receptor tyrosine kinase (RTK) signaling pathway. NCM induction of GLT1 also converges on a shared pathway with dbcAMP/EGF at the point of PI3K activation upstream of NFκB.

The majority of these studies suggest that each transcriptional regulatory element (EGF, TNFa, neurons, etc) acts through distinct but convergent pathways that lead to NFκB regulation of GLT1 gene expression in astrocytes. Areas of overlap and nodes of crosstalk between the various activation pathways, such as PI3K, present potential targets for further investigation.

**Ceftriaxone mediates changes in GLT1 transcription**

After demonstrating that the β-lactam antibiotic, ceftriaxone, caused an increase in GLT1 expression in vitro and
in vivo\textsuperscript{10}, many researchers implemented this FDA approved drug in their own research. Ceftriaxone is known to protect against adverse effects of ischemia\textsuperscript{23, 24}, attenuate addiction and reinstatement in rodent models of many drugs of abuse\textsuperscript{6, 25, 26} and be neuroprotective as a potential ALS treatment\textsuperscript{10, 27} by increasing specific GLT1 mediated GLU uptake. However, it remains unclear how ceftriaxone induces expression changes of this astrocytic GLU transporter.

Lee et al. attempted to understand how ceftriaxone treatment mechanistically increases GLT1 expression in astrocytes. Lee found that ceftriaxone mediates NFkB binding to the -272 putative binding site in the 5’ flanking region of the GLT1 promoter, and this interaction increases expression of GLT1\textsuperscript{28}. Following EGF and NCM treatment, NFkB binds to this specific site.

This induced binding is sufficient but not necessary for these factors to activate transcription, yet the -272 site is required for ceftriaxone induction of GLT1. Further insight into how NFkB binds to this genomic region will point to a pathway in which ceftriaxone intervenes to induce NFkB binding and activating transcription at this site.

Transcriptional regulation of system xCT in astrocytes

Ceftriaxone is also known to increase expression of the light-chain of system x\textsubscript{c-}, the cystine/glutamate exchanger (xCT)\textsuperscript{29}. xCT is an antiporter that exports intracellular GLU, and imports extracellular cystine in a 1:1 ratio independent of sodium but dependent on chloride\textsuperscript{31, 32}. Discussing the pathways controlling xCT transcriptional regulation can reveal targets by which ceftriaxone acts mechanistically and will demonstrate positions in the transcriptional pathway necessitating further clarity (Figure 1B).

As its name implies, xCT is critical for maintaining levels of cystine in the cell. Upon entry into the cell, cystine is reduced to cysteine, the rate-limiting precursor of the most abundant antioxidant in the brain, glutathione (GSH)\textsuperscript{33}. Maintaining redox homeostasis by GSH regulation is especially important in the CNS because the brain consumes 20% of the body’s oxygen supply and thus generates higher levels of reactive oxygen species (ROS) than other tissues\textsuperscript{34}. Therefore it is important that all proteins, with either direct or tangential roles in this redox cycle, are controlled with precision, including xCT\textsuperscript{34}.

Amino acid (AA) depletion, including but not limited to cystine, increases transcription and subsequent activity of xCT \textit{in vitro} in NIH3T3 cells, as demonstrated in work by Sato et al.\textsuperscript{35}. The induction of xCT by amino acid depletion is likely mediated by two opposing amino acid response elements (AAREs) identified in the 5'-flanking region of the xCT gene\textsuperscript{35}. This study also demonstrated that activated transcription factor-4 (ATF4) first binds the forward AARE followed by the reverse to increase xCT transcription. Loss of either site resulted in a reduced response to AA depletion, and loss of both eliminated the response. AA deprivation mediates induction of transcription, beginning with the accumulation of free tRNAs, corresponding to the deprived amino acid. The free tRNA signals by activating general control non-derepressible-2 (GCN2) kinase. The downstream target of GCN2 is eukaryotic initiation factor 2 (eIF2\textalpha{}). Important for this pathway, phosphorylated eIF2\textalpha{} acts as a transcription factor to increase gene expression of ATF4\textsuperscript{36}. In a study by Lewerenz and Maher, mouse embryonic fibroblasts with non-phosphorylatable eIF2\textalpha{}, demonstrated decreased ATF4 expression and subsequently depressed xCT expression and activity\textsuperscript{37}. Apart from GCN2, three other kinases are responsible for eIF2\textalpha{} phosphorylation\textsuperscript{36} yet it remains unclear if activation of eIF2\textalpha{} 

Figure 1. Known transcriptional regulation of astrocytic glutamate transporters, GLT1 and xCT.

A) Known transcriptional regulators and pathways of the astrocytic glutamate transporter 1 (GLT1) that act through the transcription factor NFkB. Asterisks denote sites of NFkB binding associated with ceftriaxone mediated transcriptional increases. B) A diagram showing the known pathways mediating xCT expression. (The green and red/dashed arrows indicate either transcriptional activation or repression, respectively.)
by other kinases leads to changes in xCT expression. Further investigation into the phosphorylation of eIF2α and modulation of ATF4 expression may point to novel targets responsible for the regulation of xCT transcription.

Apart from reduction of cystine to cysteine within the cell for use in protein synthesis, transport of cystine into the cell via xCT is important for the synthesis of the major endogenous antioxidant of astrocytes, GSH. Production of GSH is involved in pathways designed to alleviate oxidative stress, and the genes required for GSH synthesis, including xCT, are transcriptionally regulated by nuclear factor-like 2 (Nrf2) 38,39. This transcription factor binds to antioxidant/electrophile response elements (ARE/EpREs) and alters gene expression40,41. Sasaki et al. demonstrated that xCT transcription is activated by Nrf2 binding to four putative EpREs located in the 5′-flanking region of the xCT gene promoter42,43. Various electrophiles such as diethyl maleate (DEM) are known to induce expression of proteins that respond to oxidative stress, the Phase II detoxifying enzymes, including xCT. Furthermore, via reporter assays, the Sasaki et al. study suggests that one EpRE is responsible for DEM induction of xCT and another EpRE is required for basal regulation of xCT42. This suggests using basal or induced xCT transcriptional regulation as different mechanisms to target for therapeutic intervention.

Under normal conditions Nrf2 is bound by Keap1 (Kelch-like ECH associated protein 1), retaining the transcription factor in the cytoplasm44. In cases of oxidative stress or in the presence of electrophilic agents such as DEM, the electrophile binds the amino terminus of Keap1 causing the protein to dissociate from Nrf2. Unbound Nrf2 subsequently translocates into the nucleus where it acts to modify expression of genes required to combat oxidative stress44. The electrophilic, oxidative stress induced expression of xCT is another pathway that may provide key information regarding the mechanism by which ceftriaxone causes an increase in xCT expression.

**Ceftriaxone mediated transcriptional changes of xCT**

In addition to increasing expression of GLT1, it is reported that ceftriaxone induces expression of xCT. Lewis et al. intended to further understand how ceftriaxone offers neuroprotection against excitotoxicity via the excitatory amino acid transporters, but instead they demonstrated that ceftriaxone mediates neuroprotection via the xCT anti-porter in the antioxidant pathway29. In primary cortical astrocyte cultures, ceftriaxone induces xCT expression independent of a functionally significant increase in GLT1.

These studies suggest that ceftriaxone provides neuro- protection by increasing the levels of xCT expression leading to cystine import, and therefore increasing synthesis of neuroprotective GSH29,45. The xCT gene is the critical Nrf2 target by which ceftriaxone mediates its neuroprotective effect according to this study. In astrocytic cultures derived from xCT knockout mice, the effect of ceftriaxone, increased GSH, was greatly diminished, further supporting a role of this drug to increase antioxidant production59. Researchers still do not understand how an exogenous drug, ceftriaxone, intersects with the Nrf2 transcriptional response pathway. One study that might provide insight, demonstrates that exogenous dietary flavonoids activate PI3 kinase, which in turn increases the transcriptional activity of Nrf2 and ultimately increases GSH in astrocytes46. Therefore, PI3 kinase is a potential downstream effector of ceftriaxone in a path that leads to increased levels of xCT. More research is required to understand the exact mechanism of ceftriaxone-mediated neuroprotection. Understanding how ceftriaxone facilitates its beneficial effects, researchers and drug companies could develop drugs and treatments to directly and more efficiently target specific proteins within this pathway.

xCT expression is increased in many cells types, such as fibroblasts45, primary astrocytes45, and a hippocampal neuronal derived cell line, HT2229,47,48, to promote cell survival in culture conditions. However, in vivo loss of xCT, in the xCT knockout mouse model for example, seems to have little physiological effect 48. Although these mice did not display an increase in oxidative stress, some subtle changes were observed, such as increased levels of plasma cystine48,49. Fibroblasts derived from xCT knockout mice, however, did not survive in routine culture conditions48. In xCT knockout mice, xCT is not present during development, thus compensatory mechanisms may overcome loss of this exchanger. A conditional xCT knockout mouse model or virally mediated knockdown of xCT could provide additional insight into changes in the gene expression profile resulting from acute loss of xCT. Kalivas’ group showed that altered glutamatergic synaptic plasticity observed in rodents following chronic drug administration is restored upon chronic ceftriaxone treatment by up-regulation of GLT1 and xCT6,50,51. In this paradigm, increased expression of GLT1 and xCT attenuates drug relapse and reinstatement6. However in naïve animals with normal GLU homeostasis, ceftriaxone did not significantly alter gene expression. Consistent with these findings, it is likely that transcription of xCT is tightly regulated, and that major alterations in xCT expression occur during pathogenesis and upon major detrimental insult to the cells, such as culturing conditions or changes in glutamatergic signaling. It stands to reason that ceftriaxone mediates its neuroprotective effects in order to correct GLU homeostasis only under pathophysiological conditions, perhaps through induction of the oxidative
stress/ Nrf2 pathway.

Conclusion

Due to a high demand for fast discovery of more efficient treatments for brain disorders, drugs with FDA approval are investigated for efficacy in treatment of other diseases. However, using a drug to treat a disease for which the drug was not originally approved, calls attention to the gap in knowledge and a lack of research aimed at understanding the biological mechanism by which these drugs act. As an antibiotic, ceftriaxone was designed to kill bacteria, yet it is also efficacious in mammalian models of many central nervous system disorders. The effect of ceftriaxone as a neuroprotective agent is very striking and research points to induction of GLT1 and xCT gene expression as the primary mode of action. Increased expression may be the end result but the mechanism by which ceftriaxone adjusts transcriptional regulation of these genes remains largely unknown. By identifying the specific protein and/or pathway by which ceftriaxone mediates its effects, researchers can design new drugs to specifically interact with these targets ideally resulting in more effective neuroprotective outcomes.

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Gap Junctions in the Retina

Gap junctions, also known as electrical synapses, are porous membrane proteins that connect adjacent neurons and allow for the transmission of small molecules and electrical signals. Unlike chemical synapses, gap junctions allow for bi-directional, rapid transmission between cells and thus can effectively couple cells together both electrically and metabolically. Gap junctions are found throughout the central nervous system and have been found to play an increasingly important role in systems such as the retina, olfactory bulb, suprachiasmatic nucleus, inferior olive, hippocampus and cerebellum. While the specific function of gap junctions is highly dependent on the region in which they are expressed, a general principle of gap junction signaling is increasing signal to noise of the circuit by strengthening network synchronicity and increasing bandwidth.

Gap junctions are made up of two hemichannels called connexons that make contact between the cells. Connexons, in turn, are composed of six subunits called connexins that can form homomeric or heteromeric complexes. These hemichannels form gap junctions by tightly binding with other channels of the same (homotypic) or different (heterotypic) connexin makeup. When paired, the connexons form a short (1-2 nm) hollow bridge between the cells, allowing passage of ionic current and small molecules up to 1 kDa. A growing list of connexins isoforms have been found in mammalian retinal neurons: Cx30.2, Cx36, Cx43, Cx45, Cx50, and Cx57, with names corresponding to molecular weight. The relative permeability of channels composed of different connexin isoforms can range considerably, with Cx36 on the lower end with unitary

Dopaminergic Modulation of Gap Junctions in the Mammalian Retina

David Sprinzen

The retina is tasked with the incredible feat of perceiving light environments that range over 10 orders of magnitude. Dopamine, the primary neuromodulator in the retina, is both circadian regulated, with levels high in the day and low at night, as well as light induced and thus has been identified as the chemical messenger for light adaptation. One of the most impactful ways that dopamine is able to adapt the retina is through drastic changes in gap junctional coupling between retinal cells. By modulating the type and degree of lateral coupling at each layer of the retina, dopamine can dynamically regulate the pathway, convergence, gain, and signal to noise ratio of the visual transduction circuitry. Through this control, retinal dopamine transitions and optimizes the circuit for the cone mediated – high acuity but low sensitivity – daytime visual experience. This review will cover the mechanisms by which dopamine modulates gap junctions in the retina, the role of dopamine in each case of gap junction plasticity, and the net effect of dopamine on network coupling as it pertains to vision and light adaptation.

Keywords Dopamine, Retina, Gap Junction, Electrical Synapse, Cell Coupling, PKA, Network Plasticity, Light Adaptation

Figure 1. Dopamine Regulation of Gap Junctions in the Retina. This diagram shows the locations of gap junctions in the retina along with the effect of dopamine signaling on their permeability. The cell types of the retina are labeled such that R: rods, C: cones, H: horizontal cells, B: bipolar cells, AII: AII amacrine cells, DA: dopaminergic amacrine cells, A: amacrine cells, and G: ganglion cells. Gap junctions are represented by resistor symbols with regulation by D1-family receptors in blue and D2-family receptors in black. 1. Rod-cone coupling is reduced by DA. 2. Horizontal cell coupling is reduced by DA. 3. All cell coupling is reduced by DA. 4. Ganglion cell coupling is increased by DA. 5. Ganglion – amacrine cell coupling is reduced by DA.
conductance around 10-15 pS and Cx50 drastically higher at around 220 pS. There is increasing evidence that gap junctions can form large plaque-like structures composed of many parallel channels as well as scaffolding proteins involved with priming and modulating the connexons. By controlling the number, type and state of connexins involved, there is a wide range to how coupled the cells are. Weak electrical synapses only slightly change the excitability of a paired cell while strong electrical synapses cause action potential propagation between the cells. In this way, the degree and complexity of electrical synaptic transmission possibly rivals that of their chemical counterparts.

In the retina, all five major neuron types express gap junction proteins. Generally, electrical synapses couple a given cell type into a lateral array, mediating transmission perpendicular to the canonical, vertical flow of visual information. Much of this coupling is presumed to fine-tune and sharpen the visual signal; however, in some cases gap junctions are necessary for visual function. The ubiquitous nature of gap junctions in all animal retinas and the phylogenetically conserved makeup of the channels indicate that their use evolved along with vision, and they play a seminal role in visual processing.

Dopaminergic Modulation of Gap Junctions

Retinal dopamine is synthesized and released from a distinct class of cells called Dopaminergic Amacrine Cells (DACs). While DACs make some direct synapses onto neighboring cells, such as AII and A17 amacrine cells, the majority of dopamine signaling in the retina is likely through volume conduction. In support of this, dopamine receptors are found throughout the retina: D1 receptors on bipolar and horizontal, as well as some amacrine and ganglion cells; D2 receptors on DACs themselves; D4 receptors on rods and cones; and D5 receptors in the retinal pigment epithelium. These receptors fall into two groups of G-protein linked families: the D1 family, which includes the D1 and D5 receptors, and the D2 family, which includes the D2, D3 and D4 receptors. Importantly, activation of the D1 family leads to an increase of adenylate cyclase and cyclic AMP (cAMP) and activation of the D2 family decreases adenylate cyclase and cAMP, thus having opposite effects on their downstream signaling cascades.

In general, dopamine serves to optimize the retinal processing for daytime/bright light conditions by uncoupling the lateral interactions in the retina. The first direct evidence for dopamine modulation of gap junction coupling in the retina came from studies showing a dopamine induced reduction of dye-coupling between horizontal cells and that this process requires cAMP activation of protein kinase A (PKA).

McMahon et al. (1989) found that dopamine accomplishes this by inducing a ~5-fold decrease of the mean open time of the connexons. This change in open probability requires kinase mediated phosphorylation or phosphatase mediated dephosphorylation of specific sites on the connexin subunits, with phosphorylation sometimes causing either an increase or decrease in channel conductance depending on the site and type of connexin involved. There is evidence for a variety of signaling molecules modifying gap junction conductance in the CNS, such as nitric oxide, arachidonic acid, retinoic acid, noradrenaline, serotonin, histamine and some hormones. Furthermore, recent evidence has shown a dynamic relationship between electrical synapses and extrasynaptic NMDA receptors as well as CaMKII phosphorylation. An understanding of how these modulatory mechanisms coordinate is yet to be resolved; therefore, for the sake of this review, I will focus on the direct action of dopamine on gap junctions in the retina and how it pertains to light adaptation.

Horizontal Cell Coupling

Horizontal cells are a somewhat unique case of gap junction coupling in that they can form a highly interconnected syncytium, in which low resistance enables the cells to increase their receptive fields more than 25 times the size of their dendritic arbor. This creates a homogenous net of inhibitory interneurons whose diffuse signal can be used for surround inhibition and background subtraction within the outer nuclear layer. The primary connexin expressed in horizontal cells is the Cx57 isoform, with Cx57 knock out models causing a >99% decrease in horizontal cell coupling.

Dopamine binds to D1 receptors on horizontal cells and causes an increase in cAMP and thus activation of PKA. PKA, in turn, phosphorylates Cx57, which decreases horizontal cell coupling. Interestingly, the degree of horizontal cell coupling follows a triphasic relationship to ambient light levels, such that coupling is minimized in dark (scotopic) and bright (photopic) light conditions but significantly greater in dim light (mesopic). Because dopamine levels increase with light, endogenous dopamine reaches the horizontal cells when illumination reaches photopic levels, causing a 50% decrease in horizontal cell coupling. This system is further evident in studies of primates, giving credence to the existence of this system in human retinal physiology. Dopamine cannot work alone, however, in that other signaling proteins must encode for the decrease in coupling in very low light. The purpose of this triphasic relationship likely has to do with the type of visual information presented at each light environment. In the mesopic range, such as during dawn and dusk, the retina increases sensitivity at the expense of acuity to optimize perce-
tion of large dim objects.

The exact role of horizontal cell coupling is intricately tied to how horizontal cell signaling is utilized in the retina, which is still debated. Historically, it was believed that the inhibitory signal the horizontal cells provided was the initial surround antagonism of the retinal circuit, thus initializing the detection of spatial contrast. This dogma was recently challenged, however, when it was shown that in a Cx57 knockout that there was no loss of spatial tuning or the ability to shift to higher acuity during light adaptation. Furthermore, this study found no differences in the size of the surround inhibition of recorded ganglion cells. This suggests that horizontal cell coupling may instead be used for temporal (rather than spatial) processing, such that increases in coupling lead to longer processing time and thus increased temporal summation within the retina. More research, however, is needed to verify this possibility. What is known is that horizontal cells provide inhibitory feedback onto the photoreceptors and thus can adjust the gain control of the circuit by increasing the area over which the background subtraction is computed. Changes in horizontal cell coupling then would correspond with changes in baseline firing rate and inability to set the “tone” of the retina at a given light setting.

**Rod-Cone Coupling**

There are two classes of photoreceptors in the retina: rods, which are used in low light settings due to their high sensitivity, and cones, which transduce dynamic-colorful vision in bright environments. The retina evolved such that the rod signals piggyback onto the established cone circuitry instead of having a duplicate system for themselves. This is accomplished through the use of two main pathways: the primary pathway through the AII amacrine cell (discussed in detail later) and the secondary pathway through direct contacts between the rods and cones. This direct path between rods and cones is most evident by detection of rod-generated signal in cones, even at illuminance well below the cone threshold. Though relatively weak, rod-cone gap junctions are thought to enable rod transmission when the primary pathway is saturated, thus making a continuous transition from rod to cone vision.

Dopamine is able to signal both rods and cones through D4 receptors, which, in turn, decrease PKA and dephosphorylate the channels, correlating to a decrease in coupling. It should be noted that this is opposite to the effects of phosphorylation on the Cx57 subunits of horizontal gap junctions, signifying that the purpose of phosphorylation is highly dependent on the type gap junction involved. It is believed that the rod-cone gap junctions are composed of Cx36 on the cone side but an unknown connexin on the rod side. Two phosphorylations sites on Cx36, Ser110 and Ser276, are directly phosphorylated by PKA and likely mediate this dopamine-induced reduction.

Interestingly, Ribelayga et al. (2008) found that changes in photoreceptor coupling were dependent on the circadian rhythm of dopamine, with coupling high at night and low during the day, irrespective of background illumination. They suggest that because D4 receptors are an order of magnitude more sensitive than D1 receptors, circadian changes in dopamine levels are able to signal to the photoreceptors yet not to the horizontal cells. This ensures that during the day, bright stimulation is not shared with a saturated rod network and that during the night, the retina is optimized for low light conditions. While excessive bright stimulation at night can decrease the coupling, dim light in the scotopic and mesopic range left the photoreceptors coupled, allowing for an overall increase in the signal to noise ratio of low light transduction.

**AII Cell Coupling**

AII cells are a class of amacrine cells that mediate the primary pathway of rod signaling in the retina. AII cells receive glutamatergic signals from rod bipolar cells, then spread the signal laterally through the coupled AII cell network, which then feeds the signal onto ON cone bipolar cells through heterologous gap junctions or to OFF cone bipolar cells through inhibitory chemical synapses. Through summation of synchronous signals, AII cell coupling allows for high sensitivity transmission of the rod signal, thereby preserving signal fidelity in scotopic conditions. The purpose of AII cells seems to be twofold, however, in that during photopic lighting they convey signals in the reverse direction, from ON cones to the OFF pathway, enabling rapid inhibition that is used to detect approaching objects by some retinal ganglion cell types. This multifunctional property of AII amacrine cells highlights both the complexity of the retinal circuitry and the necessary judiciousness involved in studying the adaptive states of the tissue.

Similar to horizontal cell coupling, AII amacrine cell coupling follows a triphasic relationship to light environment, with tracer networks and corresponding receptive fields increasing in size by more tenfold in dim light as compared to dark or bright conditions. This likely enables rod transmission to retain acuity in scotopic settings, when small light sources like stars dominate the visual experience, yet increase spatial summation at twilight. By activating D1 receptors, light-induced dopamine is able to reduce the extent of coupling between AII cells, however does not have an effect on...
All-Bipolar cell coupling. Like photoreceptors, All cell gap junctions are composed of Cx36 subunits, such that Cx36 knockout removes both pathways of rod mediated vision.

Because All cells express D1 receptors that trigger an increase in PKA, it was unclear how dopamine could cause a decrease of coupling, as PKA mediated phosphorylation exhibited an increase in coupling in other Cx36 preparations. This was solved, however, when it was found that a phosphatase – protein phosphatase 2A (PP2A) – served as an intermediate step between PKA and the channel such that PKA increased PP2A activity, which dephosphorylated Cx36. In this way, both D1 receptors in amacrine cells and D4 receptors in photoreceptors are able to produce a decrease in cell coupling.

**Ganglion Cell Coupling**

Ganglion cells (GCs) are the last step of processing within the retina and must encode all aspects of visual perception. Because they represent a diverse population of morphologically and physiologically defined subtypes, how electrical synapses are utilized in ganglion cell networks is not well characterized. It has been shown that some, but not all, GCs form gap junctions with cells of the same type as well as some types of amacrine cells. The functions of these gap junctions are also diverse, with roles ranging from coding directional selectivity to increasing the signal bandwidth to the brain. Interestingly, the extent of tracer coupling between GCs does not correlate with changes in their receptive fields, which are relatively constant and determined by the extent of their dendritic arbors. Thus it is likely that gap junction mediated communication is locally defined. An emerging theme is that coupling between ganglion cells allows for synchronous firing between neighbors that may serve to enhance the saliency of visual signals.

In contrast to the other gap junctions in the retina, coupling between ganglion cells is actually increased in bright light. Hu et al. (2010) found that OFF α-GCs, which have a brisk transient response to decreases in light, were somewhat coupled in low light but increased the extent of tracer coupling by about 2-fold in photopic lighting. ON α-GCs, on the other hand, did not show any coupling. Interestingly, this coupling was found to increase the correlation of light evoked but not spontaneous activity between GCs, in accordance with the theory that these gap junctions increase signal synchronicity.

The role that dopamine plays in this process is complicated because there is evidence that both D1 and D2 receptor signaling exhibit control over it. The current model posits that D1 receptors on the ganglion cells and D2 receptors on the amacrine cells compete for control over the channels. Low levels of dopamine in dark-adapted retinas activate the high-affinity D2 receptors on the amacrine cells. Only after light adapting can the increase in dopamine release activate the D1 receptors on the ganglion cells. The net effect of this is that light induced dopamine release decreases the extent of amacrine-amacrine coupling and ganglion-amacrine coupling but increases ganglion-ganglion coupling, leading to a still overall increase in the network connectivity. This ability to differentially modulate each side of a heterologous gap junction, as well as the increasing number of connexins that are found in the ganglion cell layer (Cx36, Cx452, and Cx30.2) entail that many future studies will be needed to determine all the cases of ganglion cell gap junctions and how they are dynamically modulated.

**Conclusion**

The role that dopamine enacts in the retina, as well as its relationship to light adaptation, is intimately connected to modulation of gap junction coupling. The general design is as follows: (i) light and/or circadian signals cause dopamine to diffuse through the retina, (ii) dopamine signals receptors on different neuron classes that lead to a change in the phosphorylation state of connexins, and (iii) changes in gap junction conductivity create a retinal circuit that is increasingly modular and emphasizes cone-mediated vision. The study of this system has significant implications for understanding visual transduction, as well as the principles underlying network plasticity throughout the nervous system.

While detailing the individual cases of dopaminergic regulation of electrical coupling is important, it does not necessarily elucidate the overall effect of dopamine signaling within the retina. One way to address the integrated role of dopamine is by looking at behavioral and physiological changes in a dopamine knockout model. Jackson et al. (2012) found that using a retinal specific tyrosine hydroxylase knockout (rTHKO), in which retinal dopamine is cut by 95%, mice had a decline in both contrast sensitivity and spatial acuity measured through optokinetic tracking (OKT). Interestingly, they found that these deficits could be independently rescued using a D1 agonist or D4 agonist for acuity and contrast sensitivity, respectively. This result is consistent with the divergent control of D1 and D4 receptor signaling as applied to gap junction regulation. Since D4 receptor signaling mediates rod-cone coupling, it is possible that this has a direct role in contrast sensitivity potentially by mediating surround inhibition. Conversely, D1 receptor signaling controls horizontal cell coupling, All cell coupling, and ganglion cell coupling, thus it is possible that one or all of these mechanisms are involved with visual acuity. An intriguing
hypothesis is that dopamine signaling in the retina is separated into circadian and light-induced components, which can independently modulate retinal states based on differences in dopamine levels and receptor affinity. Resolving exactly how these signaling pathways mediate different aspects of visual processing will require more detailed physiological analysis of the effects of dopamine loss at each level of the retinal circuit.

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This paper dispelled the long held dogma that phosphorylation of connexins is negatively correlated to coupling. They showed that a phosphatase (PP2A) can serve as an intermediate between PKA and connexins, thus allowing D1 receptor signalling to uncouple gap junctions.


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This study showed that loss of dopamine induced multiple functional deficits in the retina, including decreases in contrast sensitivity and visual acuity, and that these deficits could be rescued independently by D1 and D4 receptor agonists, respectively.
Light-dependent and independent synchronization in the suprachiasmatic nucleus

Michael Tackenberg

Circadian behavior in mammals is orchestrated by the suprachiasmatic nuclei (SCN) of the hypothalamus. Within each cell of this structure, a feedback loop of gene transcription and a rhythmic regulation of action potential frequency both occur with a near-24 hour period. Cell-to-cell communication within the SCN, particularly between groups of cells in the ventrolateral portion and dorsomedial portion of the nuclei, allows for the maintenance of a coherent overall circadian phase, as well as carefully regulated phase relationships across the structure. Downstream physiological outputs of circadian pacemaking, ranging from locomotor behavior to metabolic function, can be correlated back to the phase of the SCN. Desynchrony between the external environmental cycle and the internal circadian clock, brought about through phase shifts, has been implicated in a variety of human health disorders and has been the focus of research into the properties that allow the pacemaker neurons of the SCN to adapt to new cycles. This review seeks to compile experimental evidence elucidating mechanisms that the SCN employs to maintain network synchrony of gene oscillations, firing rate rhythms, and peptide release as a free-running oscillator and in light-dependent conditions such as normal entrainment and phase shifts in light/dark (LD) cycle-specifically examining the degree to which the mechanisms underlying these conditions are similar to one another.

Keywords  Circadian rhythms, suprachiasmatic nucleus, phase-shifts, coupling signals, network synchronization

Circadian rhythms are innate, endogenous, near-24 hour oscillations orchestrated in mammals by the suprachiasmatic nuclei (SCN) of the hypothalamus. This bilateral structure is composed of roughly 20,000 neurons, each of which maintains a cell-autonomous, near-24 hour rhythm of gene expression and action potential frequency. The gene expression rhythm of an SCN neuron serves as the core of the circadian oscillation, influencing membrane potentials and action potential frequency throughout the day through circadian regulated transcription of ion channels and associated genes. Through mechanisms not yet clearly understood, membrane events in the SCN feedback to the internal gene oscillations, creating a dynamic reciprocal relationship. The complexity of these two circadian oscillations is further compounded by the need for intercellular synchronization among the neurons of the SCN in order to produce a coherent rhythmic output.

Because the endogenous circadian period of an organism is rarely exactly equal to that of the 24-hour external light/dark (LD) cycle, daily changes are required to maintain entrainment of the clock with the environment. The clock is capable of adapting to larger changes in the LD cycle, known as phase shifts, by moving its constituent oscillators to the new phase of the cycle. While both entrainment and phase shift responses are understood to be a product of re-coupling of out-of-phase oscillators, how this re-synchronization occurs remains unclear. Through some mechanism, likely signaling via peptidergic or synaptic transmission, network synchrony between SCN neurons is maintained, requiring the appropriate adjustment of firing rate and gene expression rhythms in the SCN. This synchronization occurs in the presence of light-induced signaling from the retina (such as when the oscillator is entrained to the LD cycle or shifted by a new cycle) and without external cues (causing the pacemaker to express its endogenous period, referred to as “free-running”). This review will investigate the layers of the circadian pacemaker, as well as current understanding regarding the potential factors producing network synchronization in, and coherent output from, the SCN. We will examine the mechanisms behind light-dependent adjustments in addition to coupling mechanisms used within the SCN in light-independent conditions.

The Transcription-Translation Feedback Loop

Circadian rhythms are an emergent property of the genetic oscillations that occur within each of the SCN’s constituent neurons. This transcription-translation feed-
back loop (TTFL) consists of two limbs. In the positive limb, transcription factors CLOCK and BMAL1 heterodimerize and bind to E-box sites within the promoter regions of repressor genes Period 1 and 2 (Per1, Per2) and Cryptochrome 1 and 2 (Cry1, Cry2). In the negative limb, the translated PER and CRY proteins heterodimerize and re-enter the nucleus, interfering with the binding of CLOCK and BMAL1 to E-box sites, inhibiting further Per and Cry expression.10–13

By knocking out either one of the two Per genes alone, rhythms persist, albeit with a less robust rhythm and with a shorter period.12 In peripheral tissues, such as the lung, knockouts of Per1 (PerIKO) alone are sufficient to eliminate rhythms, while PerIKO SCN slices in vitro maintain their rhythms.13 This experiment demonstrates the importance of intercellular coupling, as network synchronization in the SCN clearly confers a resistance to abnormalities within the core molecular clock - synchronization that does not occur within peripheral oscillators. The Cry gene also plays an important role in circadian function. The loss of Cry1 or Cry2 individually yields a shortened or lengthened circadian period, respectively, while elimination of both of these genes disrupts rhythms.12 Nuclear import experiments have found that PER is poorly suited to transition itself back into the nucleus without partnering with CRY, implying a chaperone role for the CRY protein.14

The CLOCK protein contains a basic helix-loop-helix (bHLH) domain that allows it to bind the E-box regions of the Per and Cry gene promoters.8 Knockouts of this protein produce animals with long-period, fragile rhythms that are prone to become arrhythmic over time.15 Like CLOCK, BMAL1 maintains a bHLH domain capable of binding to E-box regions.16 Through heterodimerization, these two proteins promote Per and Cry transcription until inhibited by the protein products of these same genes later in the circadian phase. As circadian-regulated transcription factors, CLOCK and BMAL1 regulate the transcription of many genes, referred to as clock-controlled genes (CCGs). Among CCGs are those encoding ion channels that dictate the membrane electrical properties of SCN neurons, providing a link between the TTFL and the action potential rhythms of the SCN.

Linking the TTFL to the network

Light information is encoded into the SCN network through glutamatergic signaling from retinohypothalamic tract (RHT) neurons and changes to the TTFL are elicited by calcium (Ca2+) influx occurring after light-induced depolarization of retinorecipient SCN neurons.17 This Ca2+ influx initiates signal cascades converging upon the Ca2+/cyclic adenosine monophosphate (cAMP) response elements (CREs) located in the promoter regions of the Per genes. As Per expression rates are rhythmically regulated throughout the circadian cycle, extra-TTFL inductions of Per expression levels result in a change in phase of the clock and therefore a change in phase of circadian-regulated transcription throughout the cell. This change includes circadian-regulated ion channels, which allows for the shifting of firing rate rhythms beyond initial light-induced depolarization. In retinoreceptive SCN neurons, light exposure is quickly transduced into changes in gene oscillation and action potential generation.19 The synchronization of changes in the TTFL of one SCN neuron to another, particularly from a retinorecipient cell to a non-retinorecipient cell, may be brought about independently of the membrane firing rate rhythm. Possible mechanisms include circadian-regulated peptide release, or through action-potential dependent neurotransmitter or peptide release. The signaling cascades initiated by these peptides typically converge upon CREs, much like the light-dependent signaling pathways (Figure 1). Light-induced resynchronization explains the recoupling of retinoreceptive SCN neurons after light stimulation, but fails to explain the synchronization of non-retinoreceptor cells to light cycles, or the light-independent intra-SCN synchronization that occurs under free-running conditions.

Action Potential Frequency Rhythms

Present understanding of the membrane properties of SCN neurons indicates that several types of currents contribute to changes in resting membrane potential and modulation of firing rate at different times of day.20, 21 A persistent sodium (Na+) current is observed in SCN neurons throughout the circadian cycle, which lead to depolarized membrane potentials.22 This current is counteracted during the subjective night by a leak potassium (K+) current, which hyperpolarizes SCN neurons when present, though not yet attributed to a single channel or group of channels.23 SCN neurons typically fire heavily during the day phase24, during the hours in which the depolarizing influence of the persistent Na+ current is unopposed by the leak K+ current. Action potentials during this phase are modulated by fast-delayed rectifier K+ channels, which shorten the falling phase of the action potential, decreasing the refractory period and allowing for more quickly repeated action potentials.25 Additional regulatory input comes from hyperpolarization-activated cyclic nucleotide gated ion channels, which respond to the hyperpolarized undershoot of the terminating action potential by increasing depolarizing ion flow.26 Some evidence exists for a diurnal expression of these channels, which would contribute to increased firing rate during the day.27 During the night
phase, when the hyperpolarizing influence of the K⁺ leak current is at its highest, action potentials are rare. Ca²⁺-activated, large conductance K⁺ (BK) channels expressed highly during the night phase extend the hyperpolarization phase of action potentials, further inhibiting repeated firing. As in the case of BK channels, circadian transcriptional regulation provides a means by which the TTFL can manipulate the electrical rhythms of the membrane. The membrane can also provide feedback to the TTFL through Ca²⁺ fluctuations, providing a stable, though poorly understood, reciprocal relationship between these two rhythmic layers.

**Tying the membrane electrical rhythms to the network**

Both the genetic feedback loops of SCN neurons and the rhythms in electrical properties of their cell membranes maintain many complex components that come together in careful combination to produce daily oscillations. With gene transcription oscillations capable of regulating the daily expression of ion channels contributing to action potential generation rhythms, the TTFL is clearly a source of long-term stability for endogenous rhythms that persist without external cues. Likewise, Ca²⁺-induced feedback from the membrane to the TTFL likely plays a great role in providing communication from outside the cell, either from the retina or from other SCN neurons. While electrical synapses have been shown to contribute some coupling to cells in the SCN, cell-to-cell communication is typically mediated through neurotransmitter and neuropeptide signaling. As a result, both TTFL and membrane firing rhythms must combine to produce a coherent output of peptides and/or neurotransmitters in order to provide appropriate coupling signals to neighboring neurons. It remains to be seen, however, if the neurotransmitter and peptide response initiated by light exposure during phase shifts and normal entrainment matches that of the pacemaker under free-running conditions.

**Synchronizing the Network**

The SCN is a heterogeneous structure, typically divided between the ventrolateral “core” SCN (vlSCN) and the dorsomedial “shell” SCN (dmSCN). These two regions have been shown to oscillate with identical periods, but slightly different phases (the dmSCN leads the vlSCN by about an hour). The vlSCN and dmSCN also have significantly different neurochemical characterizations. Many of the neurons of the vlSCN, which receive indirect light-exposure information from the retina via the RHT, have been shown to produce the signaling peptides vasoactive intestinal polypeptide (VIP) and gastrin releasing peptide (GRP). The dmSCN, which maintains few connections to the retina, but receives many from the vlSCN, expresses arginine vasopressin. The overall phase of the dmSCN has been experimentally shown to more closely match that of overt locomotor behavior than that of the vlSCN, particularly during shifts in LD cycle.

When cultured together, these areas maintain a phase relationship similar to that seen ex vivo, but when the two regions are physically separated, the dmSCN loses rhythmicity while the vlSCN maintains its free running period. Though this indicates that the dmSCN clearly receives input from the vlSCN, projections in the opposite direction are rare.

The importance of SCN connectivity has been demonstrated by culturing SCN slices and measuring bioluminescent reporters. After dissociation, wild type (WT) SCN neurons are more likely to drift out of phase with one another, but intact SCN maintain synchronization. Genetic
knockouts have examined this distinction further, as in one study that divided experiments between intact and mechanically dissociated SCN. *Per1* KO SCN slices from rodents were shown to be capable of maintaining rhythmicity when intact, but not when dissociated. This same effect is demonstrated with *Cry1* knockouts and shows that cell-to-cell communication within the SCN is capable of providing resistance to arrhythmicity and/or asynchrony. Because disrupting communication between SCN neurons decreases synchronization, it can be assumed that signaling between the cells is responsible for creating a cohesive and synchronized network of clock neurons. These coupling signals, however, are poorly understood.

**Putative Coupling Factors**

Several promising candidates for coupling signals have emerged, including VIP and γ-aminobutyric acid (GABA), which play strong roles in signaling between SCN neurons. VIP is expressed by about 15% of SCN neurons, while its primary receptor, VPAC$_2$, is expressed by about 60% of SCN neurons. The activation of VPAC$_2$, a G-protein coupled receptor, initiates a signaling cascade beginning with the upregulation of adenylyl cyclase and terminating with the binding of cAMP response element binding protein (CREB) to CREs on the *Per* genes, a cascade similar to that initiated by light-induced Ca$^{2+}$ influx. In other regions of the brain, VIP is capable of modulating the activity of voltage gated ion channels and it is possible that VIP fulfills a similar role in the SCN as well. Loss of VIP signaling causes hyperpolarization of the membrane of SCN neurons, likely contributing to the dampening of firing rate rhythms dependent on intrinsic excitability. Also implicated in SCN synchronization, GABA application is capable of silencing SCN neurons at all phases of the circadian cycle and induces phase shifts when acutely applied.

**Linking coupling factors to the network**

Knockouts of VIP or its receptor dampen the firing rate of SCN neurons and disrupt gene oscillations. This phenotype is rescued by daily application of VIP or a VPAC$_2$ agonist, though it remains unclear if tonic application of such a stimulus would be sufficient to restore rhythmicity. Much like VIP, daily applications of GABA re-institute rhythms in SCN cells that are out of synchronization. The release of peptides like VIP or GRP may be mediated by internal oscillations of the TTFL or in an action potential-dependent manner similar to that of GABA release. Previous experiments have shown that SCN firing rate ablation by blocking Na$^+$ channel with tetrodotoxin results in a disruption in TTFL dynamics. While this disruption may be due to a disruption in membrane potential-TTFL feedback, it may also be caused by elimination of action-potential dependent release of peptides or neurotransmitters. Further investigation is needed to separate the effects of action potential inhibition on the TTFL and on signal release, as well as to more closely examine the roles of each signaling peptide in the overall network synchronization of the circadian clock.

**Producing a Coherent Circadian Output**

When properly coupled, the circadian pacemaker is capable of producing an overt rhythmic output. The role of individual SCN neurons in contributing to the overall period of the circadian clock was investigated by crossing WT mice with those containing a homozygous mutation in the *Clock* gene, referred to as *Clock/clock*. produce a line of chimeric mice expressing varying ratios of WT and mutant neurons in the SCN. *Clock/clock* mutant neurons, which have a longer free-running period than WT neurons, were far outnumbered by WT counterparts in the SCN, the free-running period of the animal was observed to be slightly shorter than 24 hours, as would be expected in a fully WT animal. Likewise, if WT SCN neurons were outnumbered by *Clock/clock* mutant cells, the free-running period more closely matched that of a homozygous mutant animal (about 28 hours). When both cell types were represented in roughly equal number, however, a middle-ground period was observed, halfway between the just under 24 hour period of the WT and the 28 hour period of the *Clock/clock* mutant. This work provided a framework for understanding how groups of individual cells are capable of transducing a coherent output and the importance of synchronization to that output. Coupling within the SCN brings groups of neurons into phase with one another, resulting in a more unanimous, and therefore more clearly expressed, output. Inconsistencies in rhythmic output may be caused by discord between communicating cells and must be reduced through resynchronization as quickly as possible in order to produce coherent downstream rhythms.

**Conclusion**

Based upon recent work detailing the processes required to maintain synchronization in stably entrained, free-running, or phase-shifted SCN neurons, it is likely that the view of the SCN and its internal rhythms as a stable entity challenged only by changes in LD cycle is inaccurate. A more likely scenario is that the SCN, as a whole, is a dynamic structure characterized by continuous changes that are perhaps most
severe during large changes in LD cycle. Given similarities in the downstream targets of light-induced and VIP-induced signaling, specifically that of CRE activation, it appears likely that free-running intercellular coupling, normal entrainment adjustments, and phase-shift re-synchronization are ultimately mediated in the same fashion. Persistent changes in phase to a circadian clock neuron are brought about through alterations of gene expression in the TTFL, and such modifications are made through CRE activation by CREB- whether it be through calcium influx from light-dependent neurotransmission or VPAC2 receptor activation by VIP released from within the SCN.

Maintaining an accurate view of the SCN is extremely important for understanding the ways in which such a complex and heterogeneous structure is capable of producing coherent rhythmic outputs suitable for the orchestration of physiological rhythms throughout the body. With increasing focus in research today on the human health implications of (increasingly common) circadian misalignment, a more complete understanding of the SCN’s dynamic properties is an absolute requirement for improved therapies.

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Endocannabinoid regulation of nucleus accumbens glutamatergic synapses in addictive disorders

Brandon Turner

The nucleus accumbens (NAc) is extensively implicated in motivated and appetitive behaviors. Long term changes in the strength of excitatory NAc synapses is thought to underlie goal-directed learning and contribute to maladaptive behaviors in addiction. Recent reviews have highlighted behavioral similarities between drug addiction and eating disorders, including two key aspects: persistence of action despite negative consequences and chronic relapse following withdrawal. Consistent with this, the NAc has been proposed as a potential regulator of hedonic food intake. The endocannabinoid (eCB) system is a potent modulator of NAc synaptic transmission and is heavily implicated in both addictive and feeding behaviors. NAc eCB-signaling may therefore represent a common mechanism of synaptic plasticity contributing to both drug and food seeking. Understanding how eCBs are regulated in various physiological states and how they impact nuanced aspects of accumbens circuit dynamics is essential to discerning its potential contribution to these consummative disorders.

Keywords  Accumbens, addiction, plasticity, endocannabinoids, metabotropic glutamate receptors, feeding behavior

The endocannabinoid system is part of a growing list of neuromodulatory networks implicated in appetitive disorders, including eating disorders and addiction. This is likely due to its widespread expression in the central nervous system and involvement in motivation and reward processing. Endocannabinoids are lipid signaling molecules capable of inhibiting both glutamatergic and GABAergic synapses in multiple brain regions, including the NAc. Initially identified as the primary site of action of the psychoactive component of cannabis sativa, THC, Cannabinoid type-1/2 receptors (CB1R/CB2Rs) are G protein coupled receptors (GPCRs) that are localized to presynaptic axon terminals. The most common endogenous ligands, 2-arachidonylglycerol (2-AG) and N-arachydonyl-ethanolamine (anandamide, AEA), are produced de novo in the postsynaptic neuron. Activation of type-1 cannabinoid receptors, the primary isoform in the brain, decreases vesicular release probability via inhibition of presynaptic cAMP production, activation of inward-rectifying potassium channels, and inhibition of voltage-gated calcium channels. Termination of eCB signaling occurs primarily via enzymatic degradation; 2-AG is metabolized by monoacylglycerol lipase and AEA by fatty acid amide hydrolase. CB1R activation by endogenous cannabinoids can induce both transient and long lasting inhibition of synaptic transmission.

Because of the synapse-specific expression of both receptors and synthetic enzymes, eCBs can regulate the integration of synaptic information and overall circuit function. In the NAc, tuning of synaptic strength following exposure to salient stimuli is a putative mechanism for associative and reward-directed learning and is thought to underlie the generation and persistence of maladaptive behaviors. NAc eCB signaling has been implicated in behavioral and physiological responses to drugs of abuse, hedonic feeding and overeating, and is entangled with metabolic and motivational states. The purpose of this review is to frame the current literature surrounding eCB signaling in the NAc and how it contributes to synaptic plasticity underlying reward driven learning and behavior.

Nucleus accumbens structure and function

The NAc is a key mediator of hedonic reward, motivation and mood. This is accomplished via integration of information from various neocortical regions including the medial prefrontal cortex (mPFC), basolateral amygdala (BLA), and the ventral subiculum of the hippocampus (vSub), each of which is thought to contribute an aspect of stimulus salience. It is divided into two subregions: the core (NAcC) and the shell (NAcSh), each receiving unique dopaminergic and glutamatergic afferents. Similar to the dorsal striatum (dStr), the NAc consists primarily of GABAergic medium spiny projection neurons (MSNs, >90% of all neurons) with the remainder constituting multiple subtypes of interneurons. MSNs are differentiated as either dopamine type-1 receptor (D1) expressing direct pathway MSNs or dopamine type-2 receptor (D2) expressing indirect pathway.
MSNs. Direct pathway MSNs project primarily to midbrain dopamine neurons and are thought to promote movement and motivation while indirect pathway MSNs synapse primarily in the ventral pallidum and inhibit movement and motivation upon activation.

MSNs are intrinsically quiescent due to their hyperpolarized resting potential, which fluctuates between ‘up’ (-60 mV) and ‘down’ (-80 mV) states. This renders them reliant upon excitatory drive to propagate information flow through the NAc circuits. As eluded above, the mPFC, BLA, and vSub constitute the major excitatory inputs into the NAc. Integration of excitatory transmission via NAc MSNs can ultimately determine the motivational state of the organism. Additionally, inhibitory microcircuits formed by MSN-MSN or local interneuron to MSN synapses can influence NAc output by gating individual excitatory synapses or by orienting multiple MSNs toward the ‘down’ state. Ultimately, the balance between excitation and inhibition determines NAc output. Because of the integrative nature of MSNs, the strength of individual synapses is important in determining how information from multiple brain regions is processed.

Changes in NAc synaptic strength are thought to represent a cellular mechanism of reward-associated learning and memory. Such changes can be transient or long lasting depending upon the induction signal and the molecular players recruited. Long term mechanisms of plasticity, including long term depression (LTD), can induce changes in overall circuit function and influence future behavior. There are many molecular mechanisms that can result in changes in synapted strength. For example, LTD can involve trafficking and reorganization of postsynaptic NMDA and AMPA ionotropic glutamate receptors and/or presynaptic inhibition via endocannabinoid signaling. Both forms of plasticity are necessary for the generation of sensitized psychomotor behavior in rodents and are altered in the NAc following exposure to psychostimulants.

**Synaptic strength in the NAc is a cellular substrate for reward-associated learning: lessons from addiction**

NAc activity has been implicated in multiple aspects of addiction, including novel exposure to salient rewards, compulsive seeking, withdrawal, stress, and relapse in both humans and model species. Accordingly, addiction models have been the most common and effective method of studying synaptic changes in the NAc as all drugs of abuse ultimately recruit NAc circuitry. Glutamatergic synaptic plasticity in the NAc was first implicated in mediating behavioral sensitization to the psychostimulant cocaine and has since expanded to include a multitude of addiction-associated behaviors including withdrawal and the propensity for relapse. Past work has demonstrated a depression of excitatory NAc synaptic transmission following acute drug exposure, while withdrawal increases synaptic strength via postsynaptic insertion of calcium permeable, GluA2-subunit lacking, AMPARs.

The majority of evidence implicating NAc plasticity in motivated behaviors has been based upon ex vivo electrophysiological recordings which are unable to specify cell types or inputs. However, optogenetics has enabled the investigation of synapse-specific changes in glutamatergic transmission and the behavioral outcomes of in vivo NAc afferent/cell type stimulation. Recently, Pascoli et al characterized synaptic changes at BLA, vSub, and mPFC glutamatergic synapses onto both D1 and D2-MSNs in the NAcSh following withdrawal from contingent cocaine administration. This ambitious study examined basal properties as well as the ability to induce LTD using both an NMDAR-dependent and mGluR dependent stimulation paradigms. They found that mGlur5 dependent LTD was enhanced at mPFC-NAcSh synapses following cocaine exposure but was depressed at vSub-NAcSh synapses. Interestingly, they found that almost all changes in basal properties and plasticity were specific to D1 MSNs, consistent with a recent publication.

The authors demonstrated that reversal of cocaine-induced potentiation of specific synapses using in vivo induction of LTD was capable of attenuating discrete aspects of motivated behavior. Light driven mGlur5 LTD decreased discrimination of active vs inactive nose pokes when administered to vSub terminals and ablated all responding for drug when administered to mPFC terminals. These data are the strongest, to date, supporting input-specific behavioral influences and synapse-specific plasticity mechanisms. Additionally, these data support the hypothesis of synapse-specific mechanisms of plasticity underlying changes in NAc circuitry function and related behaviors. However, previous work has shown that this mGlur5-dependent plasticity likely involves eCB signaling, is impaired via sequestration of mGlur5 within the plasma membrane by homer proteins following prolonged withdrawal, and is specific for D2-MSNs in the NAc. Thus, while advances in experimental methods have bolstered the correlations between NAc synaptic strength and motivated behavior, understanding the mechanisms behind how these change in tandem requires further investigation.

**Endocannabinoids exhibit multimodal control of synaptic strength**

Discovered in the hippocampus, eCB signaling inhibits synaptic transmission in a myriad of brain regions and cell types. CB1Rs are the most abundant GPCR in the central nervous system and have been shown to function in the striatum, cortex, hippocampus, cerebellum, amygdala,
hypothalamus, and NAc. \(^3,20\) eCB-dependent plasticity can occur at either inhibitory or excitatory synapses, the expression of which varies by brain region. \(^30\) For example, hippocampal eCB-LTD is present on excitatory CA1 pyramidal cell synapses during adolescence but only inhibitory synapses in adult animals. Conversely, it is present at both inhibitory and excitatory terminals in the dStr. Because of its synaptic heterogeneity, eCB signaling can fine-tune neuronal circuits in a manner that is dependent upon presynaptic receptor expression and postsynaptic ligand production.

Cannabinoid plasticity can originate via a combination of calcium dependent and receptor-mediated mechanisms resulting in either a transient or long term depression. \(^31,32\) Induction of eCB dependent plasticity typically requires presynaptic activation paired with post-synaptic depolarization. \(^35\) This is modeled by depolarization induced suppression of excitation or inhibition protocols (DSE, DSI, respectively), where the postsynaptic neuron is depolarized for several seconds during repeated stimulation. However, presynaptic activity is also required in the presence of exogenous agonists. \(^34\) and is a means of engendering synapse specificity. Group I mGluR receptors (notably mGluR\(_5\) in the NAc) can lead to production of 2-AG by DAG lipase \(^\alpha\) (Fig 1a). \(^35\) Additionally, eCB control over synaptic transmission can be gated by various neuromodulators. Activation of D2 receptors in the dStr has been shown to block eCB signaling while A2a receptor activation suppresses the induction of eCB LTD via interactions with postsynaptic synthesis pathways. \(^32\) Thus, despite acting primarily through a single GPCR subtype, eCBs can be tailored to function at specific synapses.

**Endocannabinoid signaling in the NAc**

The inherent complexity of NAc circuitry alludes that eCB signaling may specifically function at a subset of excitatory and inhibitory synapses (Fig 1b). Indeed, CB1R expression in the NAc seems to be limited to fast spiking interneurons and excitatory terminals. \(^36\) This is in contrast to the dStr which robustly expresses CB1Rs on both D1 and D2 MSNs and exhibits MSN-MSN inhibitory LTD. \(^37\) eCB-mediated depression of excitatory transmission can be elicited by low frequency stimulation in both population and whole cell electrophysiology recordings. \(^17,18,24,38\) Low-frequency induced eCB-LTD in NAc field recordings has been shown to be independent of NMDAR, AMPAR, Group II mGluR, and D2 receptor activation but sensitive to MPEP, the selective group I mGluR antagonist suggesting a receptor dependent induction mechanism. \(^24\) Indeed, activation of post-synaptic mGluR\(_5\) by the selective agonist DHPG induces a robust eCB-dependent LTD, and these effects have been shown to be D2-MSN specific. \(^17,39\) However, D1 EPSCs are transiently depressed following mGluR\(_5\) activation or application of a CB1R agonist, suggesting excitatory afferents onto D1 MSNs express CB1Rs, akin to those in the dStr, and that D1 MSNs are capable of synthesizing eCBs via an mGluR\(_5\) dependent mechanism, although this has yet to be shown directly. \(^39\) Induction of NAc-eCB LTD has been shown to be dependent on AMP/Protein-kinase A modulation of presynaptic P/Q Type voltage gated calcium channels and the presynaptic protein Rim1\(_\alpha\). \(^17,40\)

Although eCB-LTD has not been observed at excitatory D1 synapses, it is possible that these synapses undergo transient STD following eCB release or that LTD induction requires co-activation or inhibition of other neuromodulatory receptors. Such mechanisms have been described in dStr circuits, \(^2\) while DSE remains to be reported in the NAc. Additionally, 2-AG is thought to mediate fast synaptic signaling while AEA maintains eCB ‘tone’ and facilitate long term changes, \(^4\) suggesting differences in ligand production may impact the longevity and specificity of synaptic signaling effects. This is the case in the dStr where inhibitory LTD at PV-MSN synapses is dependent upon both 2-AG and AEA and is specific to MSN down state. Conversely, inhibitory

**Figure 1. Mechanism and synaptic loci of eCB signaling in the NAc.**

A. Putative mechanism for mGluR-mediated induction of eCB-LTD, highlighting 2-AG synthesis. B. NAc excitatory synapses potentially regulated by eCB-dependent signaling mechanisms. eCB LTD induction is specific to D2 MSNs (green) in the NACC. Mechanisms of eCB/CB1R signaling at specific NACC and NAcSh synapses have yet to be determined.
LTD at MSN-MSN synapses occurs in both up and down states and is dependent primarily on 2-AG\(^{37}\). Additional work is required to determine the physiological importance of eCB signaling at excitatory D1-MSN synapses.

In addition to presynaptic action of eCBs, excitatory LTD at NAcC D2-MSNs is dependent on AEA activation of postsynaptic transient receptor vanilloid type-1 (TRPV1) receptors. TRPV1 is abundantly expressed in the periphery where it mediates responding to noxious stimuli; it is agonized by heat, pH, mechanoreception, capsaicin and endovanilloids such as AEA\(^{41}\). AEA production in D2-MSNs induces a Ca\(^{2+}\) dependent endocytosis of AMPARs resulting in LTD\(^{37}\). This is modestly blocked by CB1R antagonists but is completely blocked by inhibiting CB1R and TRPV1 together. TRPV1 activation in the presence of a CB1R antagonist is sufficient to induce this LTD, identifying postsynaptic TRPV1 activation as a novel mechanism of eCB signaling. The physiological role of TRPV1 activation in the brain has been controversial but has been strengthened by a similar study showing TRPV1-LTD in the dentate gyrus\(^{42}\). Whether TRPV1 mediated plasticity is specific for excitatory input origin is not yet known.

**Cannabinoids and reward-associated behaviors**

Cannabinoid signaling can influence both the perception and motivation for natural and drug rewards. Notably, Δ\(^{2}\)-THC, the primary psychoactive component in *cannabis sativa*, enhances motivation for natural rewards such as food and enhances NAcSh DA release\(^{44}\). Conversely, CB1R\(^{-/-}\) mice are resistant to diet-induced obesity, are leaner, and show less preference for palatable rewards\(^{45}\). Recent studies have suggested these phenotypes may be the result of eCB signaling in the forebrain, including the NAc. Deletion of CB1Rs on forebrain glutamatergic projections renders animals insensitive to THC-induced hyperphagia while mice lacking CB1Rs on GABAergic projections were insensitive to high-dose induced hypophagia\(^{45}\). Additionally, overexpression of monoacylglycerol-lipase specifically in the forebrain results in a similar phenotype to global CB1R knockouts\(^{46}\). Conversely, infusion of 2-AG directly into the NAcSh, which is associated with food intake\(^{47}\), is sufficient to elicit robust hyperphagia in rats\(^{4}\). While a comprehensive model of forebrain feeding circuits has yet to be described, the overlap of forebrain CB1R activation and NAcSh pharmacological manipulations suggests that NAc cannabinoid signaling may be an integral regulator of hedonic feeding circuitry. Consistent with these observations, the eCB system has been a recent target for anti-obesity drugs. The CB1R antagonist rimonabant is an effective treatment for animal models of diet induced obesity when administered peripherally\(^{48}\) and saw limited use clinically, albeit with detrimental side effects. Rimonabant can also impact behavioral sensitization to various drugs of abuse and can impact susceptibility to relapse\(^{49}\). IP injections of rimonabant prior to amphetamine or cocaine exposure blunts the acquisition of sensitized psychomotor behavior\(^{50}\) or consumption of a novel palatable food\(^{51}\), strengthening the role of eCBs in reward processing. Additionally, CB1R\(^{-/-}\) mice are resistant to psychostimulant sensitization suggesting impairment of NAc-affiliated circuitry. Whether CB1R blockade specifically within the NAc impairs responding for rewards is unknown. However, reciprocal data suggests that exposure to salient rewards and appetitive physiological states influences NAc eCB signaling. Several studies have shown that exposure to cocaine can disrupt the ability to induce eCB-LTD at NAc excitatory synapses\(^{37,18}\). This effect is thought to be mediated by intracellular retention of mGluR\(_{4}\) by homer scaffolding proteins, resulting in decreased eCB production. This LTD was previously shown as absent following prolonged withdrawal in NAcSh MSNs but not in the NAcC\(^{-/-}\). A clear role of eCB at excitatory NAc synapses across various drug administration time points has yet to be conclusively described.

The overlap of NAc and eCB function alludes to a joint role in the regulation of hedonic feeding. Elevated eCB concentrations are seen in the forebrain, including the NAc, following acute food restriction and are decreased following a re-feeding session\(^{5} . \) Animals chronically fed a highly palatable diet exhibit a decrease in NAc CB1R surface expression\(^{54}\). This effect may not be limited to the NAc, however, as eCBs produced in both adipose tissue and the hypothalamus are inhibited by leptin signaling and are increased in calorically replete states\(^{55}\). Diet composition is now commonly hypothesized as a likely contributor to rising obesity rates in western countries. Interestingly, rearing animals on diets lacking ω-3 polyunsaturated fatty acids, mimicking the fatty acid composition of the ‘western diet,’ blocks the ability to induce eCB-LTD in the NAc. These studies strongly suggest that forebrain feeding circuits are sensitive to metabolic state. Whether changes in eCB concentrations in the periphery directly impact NAc circuitry by mass action or whether local elevations are mediated by ascending input pathways through hypothalamic nuclei is unknown. However, consistent with its ability to specifically induce LTD at D2 excitatory synapses, increased tonic NAc eCBs could dampen D2 MSN excitation leading to an enhanced susceptibility to motivational cues\(^{52}\). Overproduction of eCBs, coupled with leptin insensitivity, could thus agonize hedonic feeding under the control of reward circuits. This is speculative, however, as interactions of eCBs within NAc circuitry and established feeding circuits or the generation of maladaptive feeding behaviors remain unclear.

**Conclusion**
The NAc functions as an integrative brain region that is dependent upon the strength of glutamatergic drive to influence motivated behaviors. eCBs within the NAc are correlated with immediate and long term changes in circuit function and affiliated behaviors. The phasic changes in eCB concentrations in the forebrain and the involvement of both eCBs and the NAc in appetitive behaviors point to a potential mechanism by which forebrain reward circuitry is modulated by metabolic state to drive reward seeking. Maladaptive changes in NAc eCB signaling may thus contribute to the development and persistence of appetitive disorders.

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Two components of reward: Wanting and liking

Converging lines of evidence support a dissection of reward into two specific components: reward consumption and reward anticipation. This component appears to be strongly influenced by brain dopamine (DA), and thus may be critical in understanding neuropsychiatric disorders associated with deficits in reward processing.

Consummatory reward is defined as the affective experience of pleasure during the consumption of a stimulus. This consummatory component has also been termed reward liking. Liking is associated with activation of specific ‘hedonic hotspots’ in the brain, including subregions of the nucleus accumbens and the ventral pallidum. In humans, reward consumption also reliably activates the ventromedial prefrontal cortex. Pleasure reactions in rodents are enhanced by opioid injections to subcortical regional hotspots; endocannabinoid and GABA-benzodiazepine systems have also been implicated in generating liking reactions. In the behavioral domain, liking can be quantified by measuring affective facial reactions to pleasant tastes (such as sucrose). These affective liking reactions that are associated with reward consumption appear to be conserved across species as homologous forms have been found in infant humans, primates, and rodents.

In contrast to consumption, anticipatory reward is defined as motivated or approach behavior elicited by the sensory properties of a stimulus. Anticipatory reward has also been termed reward wanting. Human and rodent studies have reliably shown that activation of the nucleus accumbens (ventral striatum), amygdala, and ventral tegmental area is associated with states of wanting. While opioid injections to the nucleus accumbens concurrently enhance liking and wanting responses in rodents, dopaminergic manipulations have been shown to selectively alter wanting responses toward reward-related stimuli. Blockade of nucleus accumbens DA receptors inhibits wanting behaviors in rodents, while an increase in DA transmission enhances reward wanting.

Dopamine and Incentive Salience: A Novel Hypothesis for Autism Spectrum Disorder?

Kathryn E. Unruh

Behavioral, neurochemical, and neuroanatomical research findings converge to support a model that parses reward into distinct components: reward anticipation and reward consumption. Incentive salience is the process by which stimuli in the environment become associated with anticipatory reward states through learning. The incentive salience hypothesis proposes that the role of dopamine is to attribute salience to reward stimuli and their conditioned cues, providing a mechanism to guide motivated behavior within an environment. Several neuropsychiatric disorders can be characterized by abnormalities in reward-related behavior. Addiction provides a model to study the components of reward, as its pathology leads to differential disruption of anticipatory and consummatory reward. The incentive salience hypothesis has been explicitly tested in addiction because of the known dopamine dysfunction in this neuropsychiatric disorder. Findings suggest that dopaminergic abnormalities can cause drug-related cues to become attributed with abnormally enhanced salience and therefore compulsively elicit motivated approach behaviors, at the expense of engaging in adaptive behaviors (e.g., social relationships, maintaining a job). Similarly, Autism Spectrum Disorder (ASD) is associated with decreased social approach paired with increased approach behavior toward the nonsocial world (objects). Recent research has begun to examine alterations in the functionality of the distinct components of reward processing in ASD and their underlying neural circuitry. The evidence for these abnormalities will be reviewed in consideration of a novel hypothesis for examining the pathogenesis and treatment of ASD.

Keywords reward, incentive salience, dopamine, autism, repetitive behaviors
In the behavioral domain, conditioned approach paradigms can be used to operationally define wanting as approach behavior directed toward a neutral stimulus that has been paired with the presentation of a rewarding stimulus (e.g., sucrose). Approach behavior to the conditioned stimulus, which persists even during a period of extinction (no reward delivery), supports the idea of a distinct anticipatory component of reward that can guide behavior independently of the actual consumption of the rewarding stimulus.

The neurobiological distinction between reward liking and reward wanting is supported by the findings of studies using pharmacologic lesion techniques to examine the role of brain DA in relation to reward-related behaviors. Early studies that used 6-hydroxyDA (6OHDA) lesions to induce DA depletion in rodents resulted in diminished reward-related behavior (e.g., hypophagia)\(^8\); however it was unclear whether this effect was due to a disruption in the animal’s hedonic response to reward stimuli (i.e. impaired liking) or was due to a loss of motivation (i.e. impaired wanting). Berridge et al.\(^10\) directly compared these hypotheses. While 6OHDA lesioned animals did not show altered liking behaviors (affective facial reactions) to orally administered sucrose, these animals did display significantly fewer instances of spontaneous eating than the control group. These findings led Berridge and colleagues to conclude that DA depletion resulted in a loss of the motivational significance attributed to food, resulting in a lack of approach toward food cues. In other studies, approach behavior toward sucrose is enhanced in rodents following amphetamine (a DA agonist) injection to the ventral striatum\(^6,11\), whereas behavioral (affective facial reactions to orally administered sucrose)\(^9\) and neural (concurrent measurement of firing rate in ventral pallidum)\(^11\) indices of reward liking responses are unaffected. Finally, optogenetic activation of phasic dopaminergic transmission in the rostral ventral tegmental area is sufficient to induce conditioned place preference\(^12\) and facilitate enhanced approach behavior toward food cues\(^13\).

Together, these findings support the dissociation of wanting and liking, with DA manipulations specifically affecting reward wanting behaviors without significantly altering reward liking indices. In a seminal study extending this component model of reward to humans, Knutson et al.\(^4\) developed a monetary incentive delay (MID) task and used functional imaging (fMRI) to examine differential activation of brain areas during the distinct temporal periods of the task associated with reward anticipation and with reward consumption. Participants were presented with cues indicating the magnitude of reward they could receive if they pressed a button quickly enough. Following each button press, the amount of reward they earned was displayed. In this way, the task isolated the experience of anticipating a reward (brain activation during presentation of reward magnitude cues) from the experience of consuming a reward (brain activation during the presentation of an earned reward). The authors found that ventral striatal areas were robustly activated during the reward anticipation phase, whereas ventromedial prefrontal regions were activated during the reward consumption phase. These results have been replicated in subsequent studies using similar paradigms, in both children\(^14\) and adults\(^15,16\).

**A learned component of reward: Incentive salience**

Pavlovian classical conditioning facilitates an association between specific cues and states of reward; these cues are then able to take on properties of unconditioned rewards, such as eliciting motivated behavior and activating hedonic states. When faced with an environment containing several stimuli that can elicit reward wanting, a neural mechanism for guiding motivated pursuit of reward is crucial for adaptive behavior. Goal-directed behavior is often guided by perceptual or contextual cues that become associated with reward stimuli through Pavlovian classical conditioning. These cues are able to trigger wanting of the reward they are associated with and can therefore become ‘motivational magnets’ that can independently elicit approach behavior\(^9\). This process whereby neutral cues come to acquire motivational significance as a result of learning was termed “incentive salience” by Berridge and Robinson\(^1\). Given this link between incentive salience and the DA-mediated anticipatory reward component, the incentive salience hypothesis proposes that dopaminergic transmission plays a crucial role in attributing salience to cues\(^17\). In addition, incentive salience is presumed to be a dynamic process, influenced by both learning history with a stimulus and also underlying changes in physiologic state. In this way, relevant biological states (e.g., hunger) are able to influence approach behavior. For example, a cue associated with food is more likely to elicit anticipatory reward states and associated motivated behavior during times when an organism has not eaten, relative to times when a meal has just been consumed.

Support for the hypothesis that incentive salience is mediated largely by DA functioning comes from preclinical and clinical studies that have examined differential responding to cues associated with either different amounts of response effort or different reward magnitudes. Preclinical studies indicate that DA in the ventral striatum may facilitate the ability of an animal to overcome effort-related response costs to obtain a reward\(^19\). Enhanced DA transmission is related to increased motivated behavior toward reward cues\(^19\), while diminished phasic DA transmission is associated with reduced effort expenditure in response to reward cues\(^19\). In human studies, the Effort Expenditure for Rewards Task\(^21\) (EEfRT) has been used as a reliable way to measure an individual’s abil-
ity to evaluate the costs and benefits associated with performing a motivated behavior to obtain a reward. Participants may choose to perform an easy task (30 button presses in 7 seconds with the dominant index finger) or a hard task (100 button presses in 21 seconds with the non-dominant pinky finger), given two additional pieces of information: the probability that the task will be rewarded and the magnitude of reward upon successful completion of the task. Administration of an indirect DA agonist is associated with an increased likelihood to expend more effort under low probability conditions in the EERT task, but no change in evaluation of reward magnitude. Striatal DA may provide a neurobiological basis for individual differences in effort-based decision-making; an increased propensity to expend effort under low probability conditions is also related to an individual’s DA responsivity in the absence of drug manipulation.

Differences in effort-based decision making and associated alterations in the functionality of striatal DA may provide a neurobiological model for understanding clinical disorders that are characterized by pathological alterations in goal-directed behavior. Pathological alterations in striatal DA may contribute to the types of reward dysfunction seen in a variety of neuropsychiatric conditions. Altered incentive salience has been studied in multiple neuropsychiatric disorders associated with DA dysfunction, including addiction, schizophrenia, eating disorders, and DA Dysregulation Disorder. Drug addiction is a paradigmatic example of such a clinical disorder whose pathogenesis can be related to atypical incentive salience.

Clinical significance of incentive salience: The case of addictive disorders

Contrary to expectation, prolonged drug taking (i.e., drug abuse) is associated with blunted liking responses during drug consumption. This begs the question: if exposure to drugs reduces reward liking over time, then what is responsible for the maintenance of drug-related behavior in the case of addiction?

Drugs of abuse are historically associated with DA, as many cause increases in extracellular DA in mesolimbic brain regions. Cocaine works through blockade of the DA transporter (DAT) and is often utilized in addiction studies because it is the most reinforcing substance of abuse. Positron Emission Tomography (PET) studies indicate that the temporal kinetics of cocaine-induced DA mimic or surpass that of normal phasic DA release. Conversely, the temporal kinetics of methylphenidate (MP), a DAT-blocker associated with much lower levels of abuse, are much slower than cocaine. This property may be particularly significant in the formation of addictions, as phasic dopaminergic activity is sufficient to induce conditioned place preference and increase approach behavior in rodents. PET studies also indicate cocaine uptake is greatest in basal ganglia regions, particularly the striatum. In healthy controls, both striatal DA uptake and striatal DA release (upon MP administration) are positively associated with self-reported experience of ‘high’ and increased report of craving (i.e., wanting), compared to controls. This evidence points to a dissociation between liking (experience of high) and wanting (drug craving) in addiction, illustrating a pathology that can arise through the differential disruption of reward components. Specifically, dysfunction of reward wanting may underlie drug abuse rather than increased liking responses to drug reward.

If addiction is a disorder primarily characterized by excessive reward wanting, then exposure to drug-related cues, such as contexts in which drug taking has occurred or drug paraphernalia, is likely to independently elicit drug craving/wanting and trigger drug use. In preclinical studies, neutral stimuli that are paired with drug reward acquire the ability to elicit dopaminergic increases in striatal regions and drug seeking behavior, even in the absence of drug delivery. Additionally, cocaine induces greater DA release in environments previously paired with cocaine consumption, compared to novel environments. Similarly, unexpected presentation of cocaine is associated with striatal hypactivity in drug abusers, while presentation of cocaine-related cues is associated with hyperactivity, compared to controls. While administration of MP can induce increases in striatal DA, it is not sufficient to induce self-reported or physiological craving until paired with presentation of drug-related cues. However, drug-related cues can independently elicit striatal DA release, compared to neutral cues, with larger changes in striatal DA related to higher scores on behavioral measures of withdrawal and measures of addiction severity. This evidence suggests that enhanced dopaminergic transmission to drug-related cues may underlie the persistent and compulsive nature of drug abuse.

Dopaminergic abnormalities in ASD: Substrate for reward dysfunction?

Evidence for DA abnormalities in ASD comes from neurochemical, imaging, genetic, and pharmacological treatment studies. Further, there is robust evidence for structural abnormalities in dopaminergic regions of the brains of individuals with ASD. These findings suggest a potential role for reward dysfunction in the pathogenesis of ASD.

Indirect measurement of DA function largely suggests
global activity is unaffected in individuals with ASD\textsuperscript{40-43} (but see references \textsuperscript{44,45}). However, two studies by Cohen et al\textsuperscript{46,47} have shown positive correlations between central dopaminergic function and motor stereotypes in children with ASD. Increased binding of DA has been found in the striatum\textsuperscript{48,49} (but see reference \textsuperscript{56}) and orbitofrontal cortex\textsuperscript{53} in ASD over typical controls, suggesting dysfunction may be circuit-specific, rather than general. Further, structural abnormalities of the striatum have been widely replicated in studies of ASD; the striatum and surrounding basal ganglia regions are implicated in the expression of repetitive behaviors. Robust evidence exists for increased volume of the caudate nucleus\textsuperscript{52-55}, both when controlling for total brain volume\textsuperscript{55-59} and age\textsuperscript{54}; developmentally, caudate size may increase with age in ASD, while decreasing in healthy controls\textsuperscript{59}. Further, these increases are associated with the presence of repetitive behaviors\textsuperscript{52-54,59}.

Pharmacological and genetic studies provide additional evidence for a link between DA dysfunction and behavior in ASD. Although no drugs are currently approved to treat core symptoms of ASD, two atypical antipsychotic drugs (Risperidone and Aripiprazole) with prominent dopaminergic activity have been shown to be effective in treating some symptoms associated with ASD. Randomized control trials (RCTs) of these drugs have focused on reducing challenging behaviors in ASD, with treatment groups showing significant improvements in problem behaviors (irritability, agitation, hyperactivity) compared to placebo\textsuperscript{60-63}. Additionally, treatment groups have shown reduced stereotypes\textsuperscript{64,65}. Very early pharmacological studies of the DA agonist haloperidol found that it was effective in reducing uncooperativeness and hyperactivity\textsuperscript{66,67}, autism-specific factors, including stereotypes and abnormal object relationships\textsuperscript{68}, with trend-level effectiveness in facilitating a reward-learning task\textsuperscript{68} (but see reference \textsuperscript{69}). While drug response cannot determine mechanism, antipsychotic drugs are thought to be effective due to their blockade of phasic dopaminergic activity\textsuperscript{70}, which is involved in the attribution of incentive salience. Recent genetic studies suggest the DA transporter (DAT), which is the primary molecular target for cocaine, amphetamine, and MP, may be one contributor to DA dysfunction in ASD. Hamilton et al\textsuperscript{71} characterized a de novo mutation of the human DAT (hDAT T356M) found in a male with ASD. A typical DAT regulates dopaminergic transmission through high-affinity reuptake of DA in the synapse; functional characterization indicated reduced DA influx in cells containing hDAT T356M. Further experiments revealed this is due to anomalous DA efflux in hDAT T356M cells. When hDAT T356M was expressed in Drosophila, locomotion was significantly higher in these flies than in those expressing typical hDAT. These behavioral results suggest variants in DAT, or in cellular components that interact with DAT, may contribute to abnormal DA efflux and contribute to risk for ASD-like behaviors. Together, this data suffices to implicate functional DA abnormalities in ASD.

**Reward dysfunction in ASD: Liking, wanting, and incentive salience**

Research also indicates that ASD is associated with reward dysfunction. Much of this evidence was based on the Social Motivation Theory conceptual model of autism, which posits ASD is a case of extreme early-onset diminished social motivation. This theory has led to the development of hypotheses regarding social reward dysfunction in ASD. However, in addition to diminished social behavior, symptoms of autism also include excessive interest in nonsocial aspects of the environment. Differentiating reward type into social and nonsocial may be essential for understanding reward in ASD. Furthermore, the majority of research on reward processing in ASD has treated reward as a unitary concept, rather than dissociating liking and wanting components. As will be illustrated below, considering the interaction of reward type and reward component could provide a valuable approach for interpreting and elucidating reward function in ASD.

Behavioral and intervention studies provide evidence for reward-related deficits in ASD. Individuals with ASD show impairments in learning stimulus-response reward associations\textsuperscript{72} and evaluating reward contingencies\textsuperscript{73}, compared to typically developing controls. In addition, monetary reward has been associated with faster task learning, response accuracy, and reaction time in ASD, compared to social reward\textsuperscript{74,75}; this effect is opposite of what is seen in typically developing controls. Intervention studies have shown that treatments for young children with ASD are less effective when using social reinforcers (e.g., spoken praise, facial expressions) than when using nonsocial reinforcers (e.g., tokens)\textsuperscript{76}. These behavioral findings suggest that ASD may be associated with reward-related deficits and that social reward may amplify the effect of these deficits. Thus, further pursuit of reward paradigms may reveal alterations of functional reward circuitry in ASD. While these behavioral studies of reward are helpful in documenting the presence of reward deficits, they lack a critical differentiation of specific reward components.

Parsing reward components is possible with functional imaging paradigms similar to those developed by Knutson et al\textsuperscript{4}, reviewed above. Specifically, ventromedial prefrontal cortex activation corresponds to reward liking, while activation of ventral striatal regions corresponds to reward wanting\textsuperscript{4}. Recently fMRI paradigms have been used in ASD to examine potential alterations in functional reward circuitry with respect to both social and nonsocial reward types. Several of these studies found normal activation of ventromedial prefrontal re-
gions to the presentation (i.e. consumption) of social\textsuperscript{77}, food\textsuperscript{78}, monetary\textsuperscript{79,80}, and object\textsuperscript{81,82} reward stimuli, indicating reward liking processes may be intact in ASD (but see reference \textsuperscript{83}). In contrast, reward wanting processes may be abnormal in ASD, as diminished ventral striatal activation has been show while anticipating social \textsuperscript{79,80} (but see reference\textsuperscript{77}) and monetary\textsuperscript{77,83} reward stimuli.

Motivated pursuit of reward has also been studied in ASD using the EExRT paradigm\textsuperscript{73}. Participants with ASD chose the hard task significantly more often than controls, even to obtain a small reward, suggesting insensitivity to reward contingencies. Reward probability also differentially influenced groups, as participants with ASD chose the difficult task more often than controls for both low probability and high probability reward trials. Response differences in the ASD group cannot be attributed to perseverative tendencies, as the influence of previous trials was comparable in both groups. In this study it was also found that atypical motivated pursuit of reward was related to repetitive behaviors in ASD; specifically, severity of circumscribed interests, but not motor stereotypies or insistence on sameness, significantly predicted the tendency to choose hard tasks. It is worth noting that the EExRT task has not been validated in this population and the complexity of the instructions and decision-making load may have influenced results in a population known to have cognitive and insight anomalies. However, these results provide a foundation for future research examining the contribution of reward-related abnormalities to the nonsocial symptoms seen in ASD.

The weight of reward circuitry evidence to date provides support for the hypothesis that ASD is associated with alterations in the functional response of ventral striatal regions in reward wanting processes, although it must be acknowledged that the fMRI literature is limited at this point relative to examination of reward in other disorders, and cases of contradictory evidence do exist. Evidence also suggests that individuals with ASD lack regard for reward contingencies, a finding that is consistent with deficits in anticipatory motivation. Specifically, individuals who spend more time engaging with nonsocial objects and interests appear to have a greater tendency to exert increased effort to obtain reward and are insensitive to reward contingencies\textsuperscript{73}. In typical controls, behavioral motivation has been linked to striatal DA transmission, such that increased dopaminergic activity is associated with increased willingness to exert effort\textsuperscript{23,24}. It is reasonable to consider that the observed reward-related deficits in ASD may be related to dopaminergic dysfunction, given the evidence for DA abnormalities in this population. Engaging in motivated behavior to obtain a reward is critically influenced by incentive salience, which is attributed by DA transmission. However, we currently lack studies that directly measure incentive salience in ASD, which is a critical gap in our ability to examine this reward model of ASD.

**DA-mediated reward dysfunction: A novel hypothesis for ASD?**

There is reason to consider ASD from the framework of the DA-mediated reward dysfunction that has been associated with addiction. The ASD phenotype is characterized by patterns of decreased approach to social stimuli and increased approach to nonsocial stimuli, with circumscribed interests and restricted use of objects providing clinical examples of the latter.

Increased engagement in repetitive behaviors may significantly restrict the developmental experiences of children with ASD\textsuperscript{84}. Further, these behaviors severely interfere with daily life\textsuperscript{85} and have been anecdotally reported by parents as patterns reminiscent of compulsion or addiction. The DA-mediated reward dysfunction model would suggest that altered processing of social and nonsocial reward may drive the differential patterns of approach seen in ASD.

Eye tracking paradigms have shown that children with ASD systematically approach nonsocial images at the expense of approach toward social images\textsuperscript{86-88}. This pattern of increased nonsocial image exploration has been shown to be positively correlated with overall severity of repetitive behaviors\textsuperscript{86}. The nonsocial approach hypothesis has also been tested using a behavioral paradigm thought to reflect DA-regulated anticipatory motivation\textsuperscript{23}, which indicated children with ASD are less sensitive to reward contingencies\textsuperscript{73}. This data is in line with results from fMRI studies in ASD, which show diminished activation of striatal regions during phases of anticipatory reward in individual with ASD (see above). In addition, in fMRI studies that examined activation of reward circuitry to stimuli associated with circumscribed nonsocial patterns of interest in ASD, adolescents and adults with ASD were found to show enhanced activation of reward circuitry in response to nonsocial stimuli\textsuperscript{81,82}.

The reviewed data suggests similarities between addiction and ASD, with nonsocial stimuli (e.g., objects) appearing to be capable of eliciting behavioral approach patterns and brain reward responses similar to those elicited by drug-related stimuli in drug abusers. In contrast, social stimuli may not be capable of eliciting reward responses of the same magnitude. If present early in life, these contrasting brain reward response patterns could have profound implications for experience-dependent development. Enhanced salience of nonsocial stimuli in ASD may drive preferential development of nonsocial patterns of behavior, at the expense of socially adaptive behaviors.
In addition, preclinical and clinical studies have used incentive salience paradigms to isolate specific cue-induced anticipatory responding. However, to date, studies of reward function in ASD have yet to move beyond measuring general anticipatory responses (i.e., a state of wanting). Given the evidence for dopaminergic abnormalities, coupled with differential patterns of anticipatory reward, a case can be made that the systematic examination of incentive salience processes in ASD is a critical gap to fill in future ASD research. Understanding the role of DA in the alteration of incentive salience of disorder-specific cues may therefore inform studies of the pathogenesis of ASD, as well as have profound implications for the development of interventions.

References


This seminal paper describes the development of an fMRI task that separately probes anticipatory and consummatory reward processes; the results provide functional support for the separation of reward components in humans, finding increased activation of the striatum (nucleus accumbens) during reward anticipation, but greater activation of ventromedial prefrontal regions during reward consumption.


This paper directly links individual differences in striatal dopamine to behavioral performance on an effort-based decision making task (EEFRT), indicating that increased DA responsiveness may lead to increased willingness to expend effort to obtain a reward in healthy adults.


This paper highlights the importance of cues in reward learning, finding that while phasic dopaminergic transmission is necessary to induce craving in addicts, it is not sufficient. Rather, craving is elicited by the pairing of this neural pattern with drug-related cues, indicating that indicates striatal DA increases (PET) in addicts is likely a secondary rather than primary neural response.


82. Dichter, G. S. et al. Reward circuitry function in autism spectrum disorders. Social Cognitive and Affective Neuroscience 7, 160–172 (2012). This paper was the first to look at distinct reward component responses (using a modified MID task) to ASD-specific nonsocial stimuli (objects) in individuals with ASD. Object stimuli robustly activated striatal regions during reward anticipation and ventromedial prefrontal regions during reward outcome, while monetary reward anticipation and outcome did not activate either region. This indicates that reward dysfunction in ASD is may be restricted to certain types of reward.


