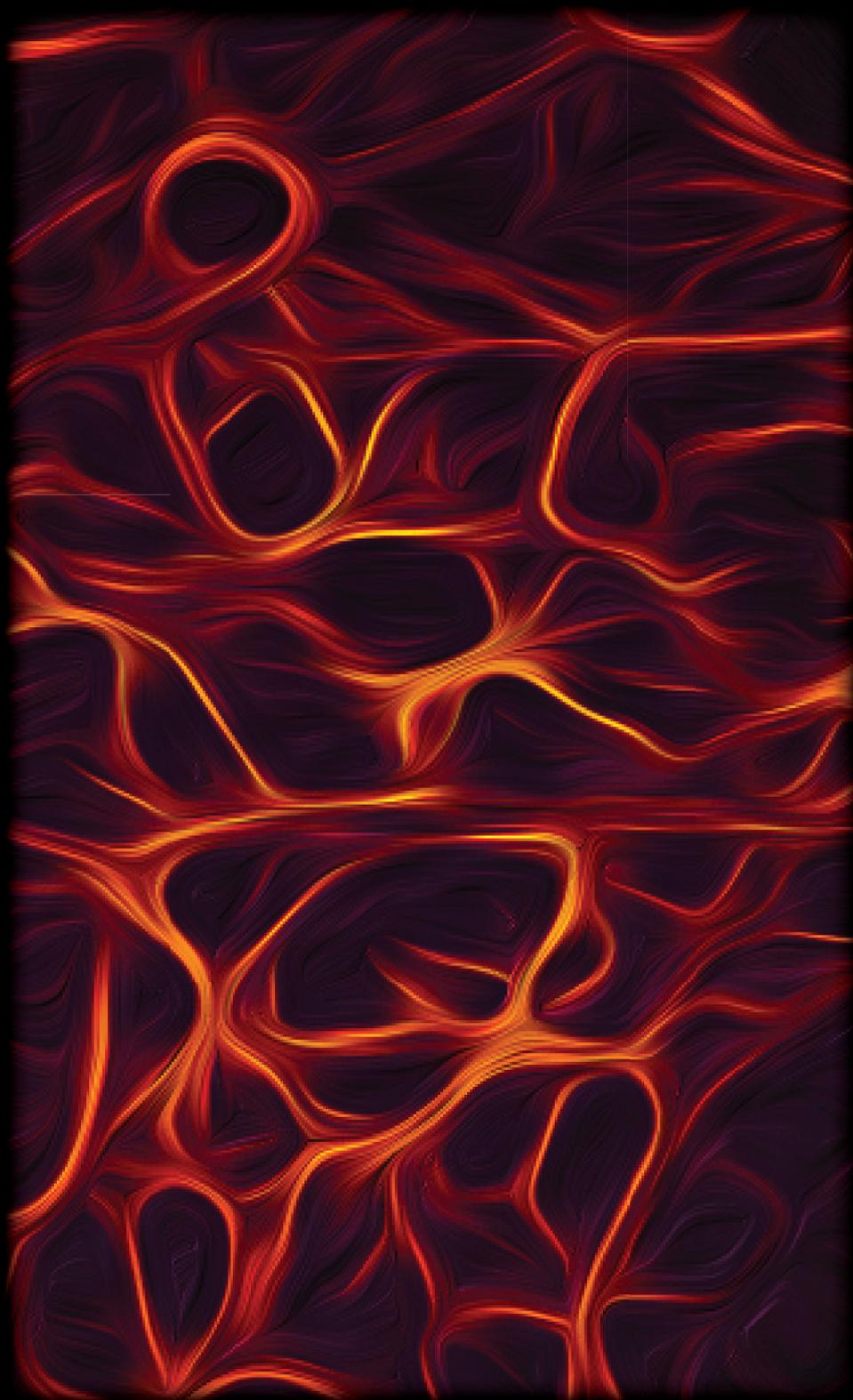


# Vanderbilt Reviews Neuroscience



Volume 8 | 2016





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## LETTER FROM THE EDITORS

Dear Friends and Colleagues of the Vanderbilt Neuroscience Community,

It is our pleasure to bring you another exciting edition of Vanderbilt Reviews Neuroscience. This year's issue contains 12 reviews submitted by the 2015 qualifying class which bring together a diversity of topics including brain networks involved in reading comprehension, the role of neural oscillations in sensory processing in autism spectrum disorders, manganese toxicity in Huntington's disease, the structure of GABA-A receptors, and the heterogeneity of neural stem cells.

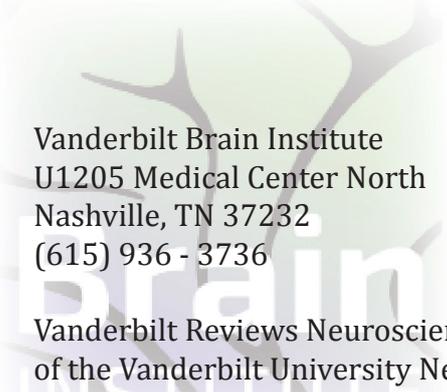
The Vanderbilt Brain Institute went through several changes in the 2015-2016 academic year. Former VBI director Mark Wallace stepped into the role of Dean of Graduate Studies, Ron Emeson took the reigns as interim director of the VBI, and several of our faculty members left Vanderbilt to advance their careers at other institutions. In spite of this major change, our program was incredibly productive this year. The Vanderbilt Neuroscience trainees published more than 50 articles, many of which appeared in prestigious journals. Following VRN tradition, we have featured a few of these publications in our Highlights and Briefs section. The Vanderbilt Brain Institute and the Neuroscience Student Organization had a successful year of outreach with the annual Brain Blast Event, the initiation of the annual Brain Awareness Week Brown Bag Series, and visits to local schools to teach developing minds about the brain. You can read more about these events in the Outreach and Education section.

This year's VRN editorial board included associate editors Shilpy Dixit and Elaine Ritter. We are grateful to this team for the countless hours spent editing reviews and communicating with the candidates and contributors to generate a high quality publication. We would also like to thank Ron Emeson and Bruce Carter for their leadership and guidance, Beth Sims and Roz Johnson for helping us assemble this year's issue, all of the 2015 candidates for their contributions, and Melissa Cooper for her beautiful cover art.

Happy reading,

Editors-in-Chief  
Kathryn Unruh & Robin Shafer





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

#### Review Process

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

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## A Message from the Interim Director of the Vanderbilt Brain Institute

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

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

Best of luck for the coming year,



Ronald Emeson, Ph.D.

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

## A Message from the Neuroscience Program Director of Graduate Studies

Dear Readers,

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

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

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

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

Sincerely,



Bruce Carter

## **An Update from the Neuroscience President**

Thank you to the editors and contributors to the Vanderbilt Reviews Neuroscience for their hard work and production of this year's wonderful publication!

The Neuroscience Student Organization (NSO) has been serving the neuroscience student community for many years, and I am proud to have served with a fantastic group of colleagues this year. The NSO officers have all worked hard on various projects to make our program even better than it is already, and I would like to take this opportunity to thank them for their dedication and highlight some of this great work.

Student opinions regarding course curriculum is an important part of our program, and our curriculum coordinators make sure that these student voices are heard. Shilpy Dixit and Eric Wilkey have worked tirelessly to continue improving our curriculum to support the needs of the program. These two were also major contributors to our new student handbook, including our new mentor-mentee contracts, which will help incoming students and their new PIs set goals and expectations, in order to help promote open lines of communication right from the start.

In addition to coursework, our students must also face the qualifying exam process, and our academic coordinators helped prepare them for this rite-of-passage hurdle. Monika Murphy, Dylan Morrow-Jones, and Brandon Moore held meetings, ran prep sessions, and scheduled mock quals all to aid students in properly preparing for their exam. These three were an irreplaceable resource to students as they made their way through this process.

A vital part of any education is to give back, and the neuroscience program works hard to see that this happens. Rose Follis and Stephen Bailey have thrived in the role of outreach coordinators. These two planned a tremendous Brain Blast event, coordinated brown bag talks for Vanderbilt staff to learn about neuroscience topics, organized student lectures at the Brentwood library for the adult community, and put our students into Nashville public schools to teach classes on the science of the brain, promoting the VBI name and getting us into the community to give back.

Every year, our program holds a retreat, and this year it will be held at Arrington Vineyards. Alyssa Lokits, our retreat coordinator, has done a fabulous job of planning and organizing this event at a great new location! This event is a great time for the whole program to come together, learn about recent updates in each other's research, celebrate our accomplishments and achievements, and relax and have some fun, too!

While research and lab work is a huge part of our lives as students, it is important to balance all of that hard work with some relaxation and socialization, too. Allyson Mallya has done a fantastic job as our social coordinator this year. She has organized some great social events for us as a neuroscience community, providing students with some time to gather and unwind from some long days in the lab.

Thank you to everyone for all of your hard work and dedication to the NSO and the Vanderbilt neuroscience community. It has been a fantastic year and I am excited to see what wonderful changes and additions the next year, and next group of NSO officers, brings!

LeAnne Kurela

## Brain Blast | Community Outreach

Community outreach is a key mission of the Vanderbilt Brain Institute. 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 Committee, which consisted this year of PhD students Rose Follis, Gabby DiCarlo and Stephen Bailey.

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.”

The VBI’s signature outreach event, 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. 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. This year, the VBI partnered with Tennessee State University to host Brain Blast at new location on TSU’s campus. Graduate students and research staff from VBI-affiliated labs, including neuroscience graduate students, facilitated interactive booths. Booth activities 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. This year saw the return of Brown Bag lectures, featuring talks by PhD students on dyslexia, autism and sensory processes. As scientists, we have a responsibility to share our work with the people who fund research, but community engagement and outreach generates interest in science and an appreciation for what scientists do. We hope to reach future scientists with our outreach efforts.



## RESEARCH HIGHLIGHTS

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### *2-arachidonoylglycerol signaling impairs short-term fear extinction.*

**Hartley, N. D., Gunduz-Cinar, O.,  
Halladay, L., Bukalo, O.,  
Holmes, A., & Patel S.**

Abnormal fear conditioning responses have been heavily implicated in neuropsychiatric disorders. Specifically, abnormal fear extinction is associated with stress disorders including post-traumatic stress disorder (PTSD).

While acute neural response to traumatic stress serves an adaptive function by conditioning the organism to avoid potentially harmful stimuli, in pathologic conditions, the fear response can be generalized to contexts and cues that are not associated with the trauma. This is thought to be the consequence of deficits in fear habituation, extinction learning, or enhanced sensitization of fear.

Endogenous cannabinoids (eCBs) are believed to play a role in fear extinction by mediating presynaptic neurotransmitter release through retrograde synaptic signaling. 2-arachidonoylglyceride (2-AG) is an eCB that is expressed in brain regions associated with PTSD such as the amygdala.

This study aimed to determine whether 2-AG is involved in short-term extinction learning of a conditioned fear response. The pharmacologic agent JZL184 is a monoacylglycerol lipase inhibitor that blocks degradation of 2-AG and was used to increase 2-AG levels in the brains of mice. For cued fear conditioning, mice were placed in a conditioning chamber and were conditioned to associate a tone with a foot shock. Freezing behavior was measured as the fear response. Short-term cue dependent extinction learning took place 24 and 48 hours later in a separate

conditioning chamber that was distinct from the condition chamber used for acquisition. Mice were administered intraperitoneal vehicle or JZL184 prior to the sessions, and during the sessions they were exposed to several repetitions of the tone without a paired foot shock. JZL184 treated mice showed impaired extinction learning (persistent freezing in response to the tone) compared to the vehicle treated mice; however, by the third extinction session the groups did not differ. This indicates that elevated 2-AG interrupts short-term extinction learning of a cued fear response within a sensitive window. Cue conditioned fear and extinction findings were replicated with injections of JZL184 directly into the amygdala indicating that heightened 2-AG acts in the amygdala to disrupt extinction learning of a cued fear response.

Acquisition of context dependent fear response involved exposing the mice to foot shocks in the conditioning chamber without the paired tone. Extinction of the contextually conditioned fear response involved placing the mice in the same conditioning chamber 24 and 48 hours after acquisition. Mice were administered vehicle or JZL184 before the sessions. JZL184 treated mice showed deficits in extinction of the contextually learned fear response. Contextual fear conditioned mice that were treated with JZL184 also demonstrated increased fear sensitization to unconditioned tones in a novel context indicating that increased 2-AG levels contribute to increased fear sensitization.

These results provide insight into the role of 2-AG in the persistence and generalization of a fear response. These findings indicate that high levels of 2-AG can contribute to the generalization and impaired extinction learning of a fear response. Since these processes are associated with stress disorders such as PTSD, this study has important implications for the understanding and treatment of these disorders.

## RESEARCH BRIEFS

***Relative contributions of visual and auditory spatial representations to tactile localization.*****Noel, J. P., & Wallace, M.**

Identifying the spatial location of tactile stimuli requires coordination between multiple reference frames in order to form a coherent percept. This often involves somatotopic reference frames that encode the location of the stimulus on the body and body-centered reference frames that encode limb position. Visual and auditory stimuli also provide important spatial cues in retina and head centered reference frames, respectively; however, little work has focused on the influence of visual and auditory information in spatial localization of tactile stimuli. In this study, participants were presented with a pair of vibrotactile stimuli (one on each ankle) that had a stimulus onset asynchrony ranging from -200 to 200ms (negative is right leading and positive is left leading). Participants were asked to identify which ankle received the stimulus first. Participants performed the task in two different postures (legs parallel and legs crossed) and in two different sensory conditions (normal and deprived). Sensory deprivation consisted of auditory, visual, or audio-visual deprivation. Results replicated previous findings of reduced performance in the crossed legs posture. There was an effect of sensory condition indicating that auditory and combined audiovisual deprivation, but not visual deprivation, resulted in poorer performance. There was also an interaction between posture and sensory condition driven by reduced performance when legs were crossed in the auditory and audiovisual deprivation conditions. These findings indicate that tactile spatial sensitivity is dependent on auditory and combined audiovisual information even when that information is not related to the stimulus.

***CHIP Is an Essential Determinant of Neuronal Mitochondrial Stress Signaling.*****Palubinsky, A. M., Stankowski, J. N., Kale, A. C., Codreanu, S. G., Singer, R. J., Liebler, D. C., Stanwood, G. D., & McLaughlin, B.**

Dysfunction in ubiquitin ligases – which are responsible for identifying damaged proteins for degradation – and mitochondrial dysfunction have been implicated in neurologic disorders including Parkinson's disease and Alzheimer's disease. It is believed that ubiquitination is associated with mitochondrial function. The C-terminus of the HSC70-interacting protein (CHIP) has been shown to be involved in neuroprotection by promoting protein degradation and refolding, and CHIP haploinsufficiency results in severe motor deficits. This study characterizes the mechanisms through which CHIP responds to mitochondrial damage and stress. Post mortem tissue samples of CHIP haploinsufficient mice had increased protein oxidation, lipid peroxidation and energetic stress early in development, as well as impaired antioxidant defense mechanisms relative to wild type mice indicating that CHIP is involved in responding to oxidative stress. Healthy neurons cultured under oxygen-glucose deprivation (OGD) to simulate metabolic stress showed increases in CHIP trafficking from the cytosolic and perinuclear regions to the mitochondria. The integrity of CHIP deficient neurons did not appear to be different from wild type neuron cultures under unstressed conditions, but under OGD conditions, CHIP was necessary for maintaining mitochondrial integrity. Consequently, under OGD conditions, CHIP deficient neurons had higher rates of cell death. These results indicate that CHIP plays an important role in mediating energetic processes in the cell by regulating homeostasis of the mitochondria. These findings have important implications for the study of many neurologic disorders associated with oxidative stress and mitochondrial dysfunction including ischemia, Parkinson's disease, and Alzheimer's disease.

## On the Cover

To create it, I fluorescently labeled GFAP in a whole-mount retina to show the cytoskeleton of astrocytes, then mounted the tissue and imaged it on a confocal microscope. I then changed the brightness, contrast, and color composition of each image within the z-stack to create more depth. Through various photoshop techniques, I then added additional colors and created the illusion that the image was an oil painting.

- Melissa Cooper



# A Network Basis for Reading Comprehension

*Stephen K. Bailey*

## Abstract

Comprehending written text is a complex act that arose only recently in human history. Skilled reading involves interactions between brain areas that support skills specific to word reading (occipito-temporal cortex), language (perisylvian cortex) and domain-general executive functioning (frontal cortex). Recently, researchers have used functional magnetic resonance imaging in individuals at rest to model the intrinsic relationships between these brain areas. One widely used technique is graph theory, which treats each brain area as a single “node” in the larger brain network and temporal correlations with other nodes as “edges”. Graph theory analyses have shown that the brain has a modular structure that facilitates efficient information transfer between any one area and another through highly connected hub regions. While these insights are important from a qualitative perspective, the utility of these metrics in relating to behavioral indices of reading is largely unexplored. However, applications in other research fields, such as neuropsychiatric disorders, suggest that graph theory may be useful for studying individual differences in cognitive functioning. Reading comprehension, which relies on information integration within and between multiple brain regions, may be particularly amenable to this type of network analysis.

**Key Words:** *fMRI, resting-state, brain organization, reading, comprehension, language, hubs, network*

## Introduction

Comprehending text is a necessary skill in modern civilization, but a large percentage of people struggle with it. In Nashville, for example, 1 in 8 adults is illiterate. Often, reading difficulty is caused by poor word recognition or trouble mapping word sounds (phonology) onto symbols (orthography). However, even when individuals are able to recognize words, they may struggle with comprehending the text’s primary message. While several skills important for fluent comprehension have been identified, including phonological awareness, vocabulary and attention, diagnosing and assisting poor comprehenders remains difficult. This has motivated researchers to investigate the biological basis for reading, which may provide insight into whether fluent comprehension is more than simply the sum of its parts.

Much of our current understanding of the brain basis for language stems from a scientific tradition of localization. Nineteenth century phrenologists culled insights about personality and aptitudes from skull structure; twentieth-century neurologists used brain damage to infer cortical

specialization. More recently, scientists have used functional magnetic resonance imaging (fMRI) to map brain activity during cognition, from the simple (finger tapping) to the sophisticated (language comprehension). In the case of reading, neuroscientists have converged on a set of regions constituting a basic reading network in the left hemisphere, including occipito-temporal for word recognition, temporo-parietal for semantic processing and inferior frontal areas for articulation and encoding<sup>1</sup>. However, in the case of comprehension, it is clear that brain areas do not act on their own but in concert. Damage to the white matter tracts connecting them can have as devastating consequences as damage to the cortical areas. Indeed, even damage to areas that are not considered primary to language can have a debilitating effect: right hemisphere lesions can affect how well an individual can understand the metaphorical meaning or emotional salience of text<sup>2,3</sup>.

The appropriate level of investigation for understanding reading comprehension (RC), rather than the cell, circuit or gyrus, may thus be the

network of brain areas. In this review, we discuss network theories of reading development and then explore how one approach to network analysis -- graph theory -- has been used to relate net-

work properties to behavioral indices of cognition. Finally, we discuss the future of these approaches and the potential benefit they may confer.

## Cortical Specialization in Reading Development

In the United States, children are taught to decode words between the ages of four and nine years. This is a time of major developmental changes in the brain, including synaptic pruning and  $e^{-4}$ . Reading development thus occurs during a period of both active, intrinsic development and structured, extrinsic instruction. The brain areas responsible for fast and efficient word decoding may become specialized through a process of interactive specialization, in which intrinsic developmental processes and experience collaborate to form the mature, skilled reading system <sup>5,6</sup>. The theory is built on the Hebbian maxim that “neurons that fire together, wire together”, with the brain being considerably more plastic during this developmental period than it is later in life <sup>7</sup>.

An important step in building reading skill is learning to recognize words by sight (e.g. to be able to quickly and automatically associate the sound /kat/ with the letters C-A-T. This is mediated through a portion of the fusiform gyrus: neural activity appears to become increasingly specific to words as individuals become better at recognizing words <sup>8,9</sup>. Consistent co-activation between early processing areas in the visual cortex and the fusiform gyrus may create long-lasting correlations between neuronal activity in the two areas. Failure to develop this sensitivity to word stimuli may be one of the causes of poor reading <sup>10</sup>.

This process of interactive specialization may cause lasting changes to connectivity even when subjects are not reading. Evidence for this comes from resting-state fMRI, which does not require individuals to complete tasks but instead uses spontaneous neural activity from the fMRI signal to construct networks. Koyama et al. found that many reading-related nodes had overlapping connectivity with the left inferior frontal gyrus and left middle temporal gyrus, both nodes that are important for skilled language use <sup>11</sup>. A follow-up

study comparing IQ-matched children and adults found similar patterns: better readers in both groups showed increased connectivity between the inferior frontal gyrus and the middle and superior temporal gyri, as well as between the precentral gyrus and motor areas <sup>12</sup>. In adults, positive correlations were found between reading ability and connectivity between the visual word form area and phonological processing areas; in children, however, this correlation was weaker and negative, suggesting that the visual word form area becomes more integrated with experience as well as skill. Reading intervention also exerts an effect on connectivity patterns. Dyslexic adolescents who received reading remediation had higher correlations between the visual word form area and the right middle occipital gyrus than did control participants <sup>13</sup>. This connectivity also correlated with spelling and single-word reading scores.

However, word recognition skill is insufficient to explain individual differences in comprehension <sup>14,15</sup>. That is, comprehension requires more areas of the brain than do those of more basic reading skills. Xu et al. reported that in single-word reading tasks, neural activity is largely confined to areas near the visual system in fusiform regions, whereas in sentence and passage comprehension, readers utilize a broader array of brain regions <sup>16</sup>. In an fMRI scanner, participants read a stream of words, followed by a stream of sentences and finally a full narrative. Across all three levels, there was activation in perisylvian language areas. Sentence processing correlated with increased activity in the bilateral temporal poles. However, when the same sentence stimuli were presented within the context of a narrative, the authors found large activations across both hemispheres, including the precuneus, medial prefrontal and dorsal TP and occipital cortex.

Other reports corroborate these larger activations: Cutting et al. used a sentence comprehension

task which elicited bilateral temporal lobe activation (left > right) and greater occipital lobe signal, as would be expected from increased language and visual load. Thus, sentence comprehension relies on a core set of extended language regions above that required for words<sup>17</sup>. Consistent with theory, as readers become more skilled, patterns become more focused and sharply defined; in contrast, those who contin-

ue to struggle with reading (i.e. dyslexia) continue to show a more diffuse pattern of activity<sup>18</sup>.

To investigate interactive specialization in comprehension and other multifaceted behaviors, looking at single connections between areas of the brain may not be sufficient. Instead, the properties of the larger system of brain areas must be accounted for.

## Resting-State Networks

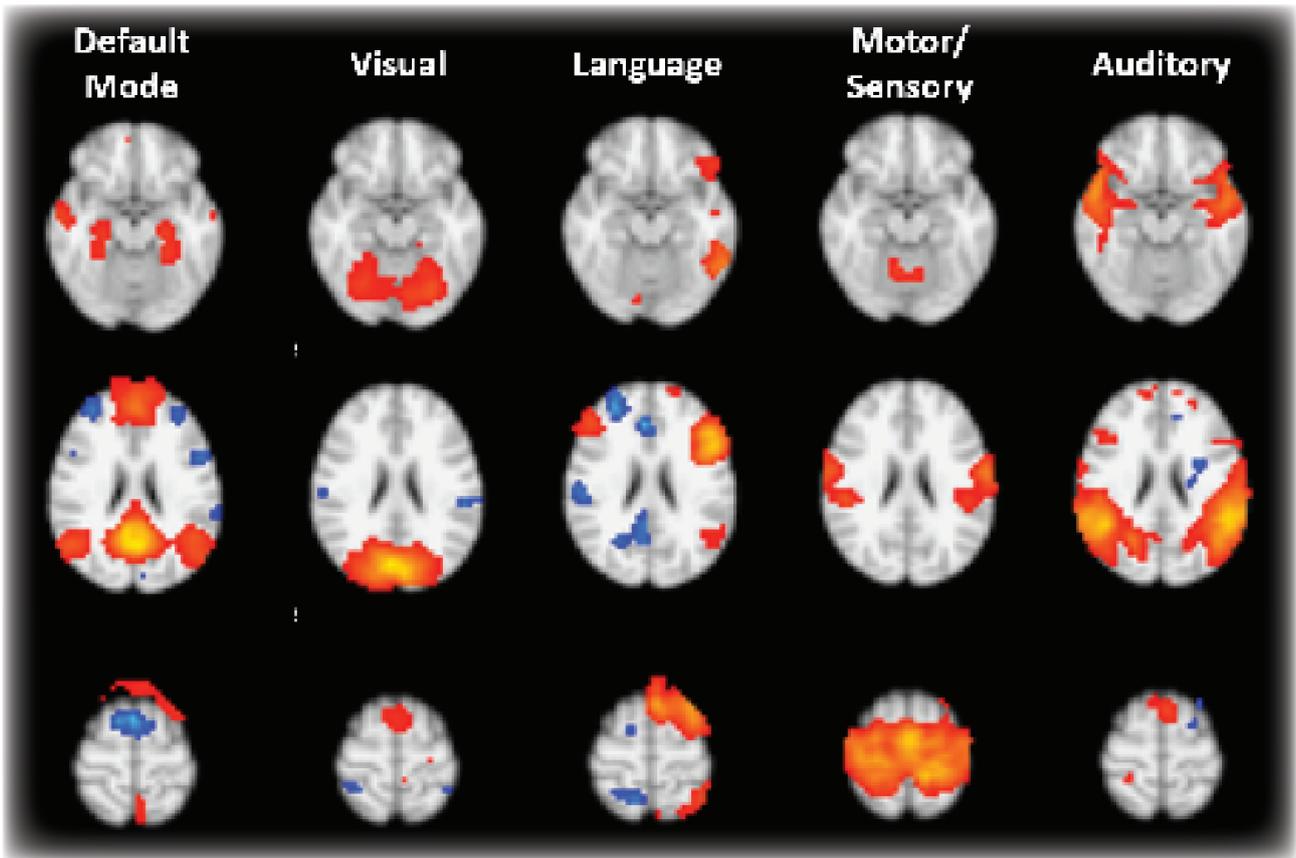
In 1995, Biswal et al. discovered that portions of motor cortex that were active during tasks were also correlated with other areas at rest, suggesting that there was an intrinsic connectivity between these areas<sup>19</sup>. However, in the excitement of the early years of fMRI, these non-task-related findings were of little interest. In 2003, Greicius et al. found that the “default mode network”, a set of brain areas that was commonly seen anti-correlated during tasks, was found to be active at rest<sup>20</sup>. Since then, scientists have been actively identifying and characterizing intrinsic resting-state networks (RSNs) that can be reliably found in individuals at rest. A number of RSNs have been identified which may underlie cognitive function, including language<sup>21,22</sup>, visual perception<sup>23</sup>, motor functioning<sup>19</sup> and executive control<sup>23,24</sup>. Although there can be significant variability between and even within scans<sup>25</sup>, these functional connectivity findings are robust and have been repeatedly found in large scale datasets.

Beyond simply identifying these networks, scientists have used graph theory to analyze how the brain areas that make up these networks act in terms of the entire system of brain networks (sometimes called the connectome). In graph theory, each brain area is a single “node” in the larger brain network and temporal correlations with other nodes are “edges”. In RS-fMRI, similar cortical areas (e.g. Brodmann areas) are typically assigned to be nodes. In some cases, the entire brain is input into the analysis, whereas others use a more targeted, seed-based approach<sup>26</sup>. Edges are often weighted, meaning areas that are more highly correlated carry a greater connectivity value. Next, an algorithm is applied to the matrix of correlations to distinguish sets of nodes that

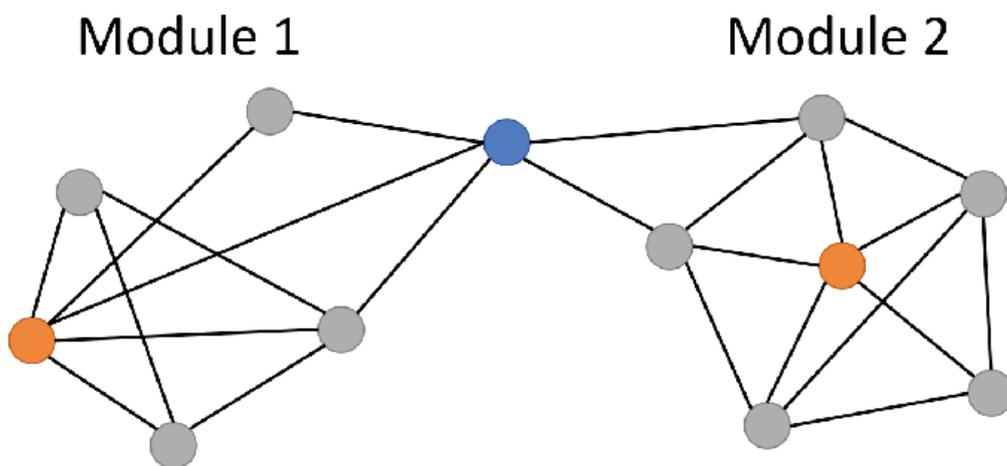
are more highly connected to each other than to other areas of the brain. Finally, first- and second-level properties of the networks are derived.

The decision of which regions to include as nodes is critical. Typically, one of several approaches has been used to identify nodes: anatomical parcellations based on an atlas<sup>27-29</sup>; individual voxels<sup>30</sup>; functional ROIs from either a priori hypotheses or task-based activation<sup>31</sup>; or an algorithm that parcellates the brain independent of function or anatomy<sup>32</sup>. Differences in these methods can affect the RSNs identified. At high resolutions (e.g. voxel-level correlations), there is a greater chance of spurious correlations causing noise in the data; at lower resolutions, the time series for a region may blend multiple functional regions, creating a composite that does not truly reflect any of the underlying areas.

Graph theory provides several metrics for consideration, of which we discuss three: node degree is the number of connections a single node has<sup>33</sup>. Nodes with higher degrees are thought to communicate with a greater number of nodes than others; networks with a higher average degree are thought to be more densely connected. Path length is the minimum number of nodes that must be passed to connect one node to any other. A completely random network will have a relatively low path length; a completely regular one will have a high path length. Finally, the participation coefficient is the degree to which a node participates in networks other than its primary one. These properties have been used to find a number of interesting properties about the brain. Developmentally, RSNs exhibit increasing functional correlation across the life span<sup>34, 35</sup>.



**Fig. 1.** Examples of the visual, auditory and default mode resting-state networks. Independent component analysis was performed in 16 adult subjects using resting-state fMRI. degree of all nodes.



**Fig. 2.** Schematic for a network with two modules. The blue node is a hub region critical for connecting modules 1 and 2. The orange nodes have the highest degree of all nodes.

Properties of these RSNs, including density of connections, along with their locations and changes with development are of primary interest<sup>36-38</sup>. RSNs in children are more greatly constrained by proximity than in adults but are functionally organized: visual system regions, for example, form their own community, as do auditory regions and

executive control regions<sup>24</sup>. Several studies report that the brain takes on a modular structure, consisting of many densely intra-connected networks<sup>37-40</sup>. These modules are connected to each other by a smaller number of regions, dubbed “rich clubs” or “hubs”, that may facilitate the passage of information from one module to another<sup>41,42</sup>.

## Application of Graph Theory

These findings have informed our understanding of the brain’s large-scale organization. Rather than having a set of tracts that connect all regions, these findings suggest the brain is organized complexly but efficiently in a “small-world” architecture. This has emphasis on the importance of certain regions to fulfilling cognitive functions: in one study with lesioned patients, Petersen et al. predicted how severe a lesion would be based on its proximity to these hub regions<sup>43</sup>. They found that lesions on areas that were not densely connected showed less extended impairments than regions that were highly inter-connected in RS-fMRI. Other psychiatric disorders have found disruptions in network properties. Lord et al. found that while whole-brain metrics of modularity were similar across individuals with depression, RSNs “reorganized” in individuals with depression<sup>44</sup>. Thus network properties may be important for explaining individual differences in neuropsychiatric disorders or cognitive skill, such as comprehension.

Relatively few studies have been done relating network methods to reading ability. Despite the changes in connectivity between areas, reading-related regions such as the fusiform gyrus, angular gyrus and inferior frontal gyrus, do not create one distinct RSN, but are members of separate, more primary RSNs<sup>45</sup>. Finn

et al. compared graph theory metrics of dyslexic and non-impaired readers using graph metrics<sup>46</sup>. Dyslexic readers showed divergent activity in visual association and prefrontal attention areas as well as increased right-hemisphere connectivity. Differences were persistent across both adult and children readers, suggesting that network metrics are relevant for reading. Another study in which participants were asked to silently read a sentence that had either a semantically congruent or incongruent ending, showed that graph theory metrics were relatively stable overall, but some circuits were sensitive to semantic properties<sup>47</sup>. These initial investigations suggest that network metrics may be an appropriate tool for investigating brain regions that coordinate to accomplish RC.

According to the view of interactive specialization, an integrative ability like RC would be more highly connected than localized skills. The multi-RSN robustness, or how well-maintained the network is after deletion of a node, will measure how strongly inter-connected the RSNs of interest are to each other<sup>39</sup>. An important consequence of these hypotheses is that, if there is a biological substrate underlying RC, we may have another window into predicting how well a child will continue to develop as a reader, especially during the periods of rapid development in primary school.

## Prediction of Future Reading Skill

One promising area of application is that of prediction. Machine learning techniques such as multivariate pattern analysis have previously been used to classify individuals with developmental and neuropsychiatric disorders, including individuals with dyslexia<sup>48-52</sup>, autism and depression<sup>44</sup>. These

techniques can incorporate multiple sources of information, such as network properties, to build the optimal model for predicting whether a dataset belongs in one group relative to another<sup>53</sup>. This has been done with anatomical and functional data<sup>51,54</sup>. Network properties may be even more likely

to contain information about an individual's likelihood of succeeding in school than other metrics.

However, the neurobiological basis for these RSN properties is still under investigation. While these connections do appear to be plastic and mediated by experience, they are not necessarily caused by new synapses. Several studies using diffusion-weighted MRI (DW-MRI) suggest that functional connectivity represents more than simply direct synaptic connections. DW-MRI uses water movement to model the white matter tracts within the brain. At high resolutions, it provides a coarse approximation of the human connectome -- the total connections in the human brain<sup>55</sup>. Honey et al. investigated whether functional connectivity can be predicted from structural connectivity<sup>25</sup>. Five subjects underwent DW-MRI and RS-fMRI scans,

and brains were parcellated at both a high resolution (998 cortical regions) and low resolution (66 cortical regions). They found that, while structurally connected areas are typically functionally connected as well, the inverse is not true. Areas that were closer together were also more highly functionally connected, possibly due to structural cortico-cortical projections.

More work, including longitudinal studies, certainly needs to be done to understand the complex processes. One of the outstanding questions in the field, though, is what (and even whether) these properties have a neural or psychological basis. While it is likely that RSNs reflect a long history of co-activation between brain areas<sup>56,57</sup>, it is less clear what individual differences in a specific metric might mean.

## Concluding Remarks

The network analysis of RS-fMRI could be especially useful for research on hard-to-study populations. Unlike task-based fMRI, which requires many trials and multiple baselines, RS-fMRI can be done in as few as five minutes. Additionally, since no task is being administered during these scans, interpretation is not subject to differences in subject aptitude or familiarity. For example, a task-based study investigating text comprehension might ask participants to read a passage that has been tightly controlled for difficulty. However, since task difficulty influences neural activity<sup>58</sup>, individuals with greater or lesser cognitive ability, or even executive function, would likely show differing patterns of activation not related to the task studied.

A critical issue in the network analysis of fMRI data is motion correction. Even small amounts of motion can induce spurious correlations in the data, obscuring the actual network properties being studied<sup>59,60</sup>. These spurious correlations have caused several leading groups to reinterpret their findings in the last several

years. New techniques for correcting data are making rapid progress in improving upon this problem.

In conclusion, researchers have uncovered a great deal about how the ability to read develops at both a behavioral and neural level, and new network analysis approaches hold great promise for discovering even more. In particular, they may help uncover how the different networks of regions involved in reading are able to coordinate and interact throughout the process of reading, as well as how they develop over time. A better understanding for the network basis of comprehension will help identify children and adults who may have intractable reading difficulties, and it could ultimately result in better screening procedures. However, the neurobiological basis of these network properties is still relatively unknown as are how they directly relate to well-established behavioral indices. Future work will be important for addressing which properties of the networked brain are most salient for psychological study.

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# A Pathogenic Role for Manganese Dysregulation in Huntington's Disease

*Miles R. Bryan*

## Abstract

Manganese (Mn) is an essential metal for almost all biological systems, serving as a co-factor for numerous biologically indispensable enzymes and capable of activating many critical cell signaling pathways. Recently, several studies have examined a potential link between Mn dysregulation and Huntington's disease (HD), a devastating neurodegenerative disorder caused by death of medium spiny neurons in the striatum. Several HD endophenotypes (i.e. glutamate excitotoxicity, increased urea and nitric oxide toxicity, increased reactive oxygen species (ROS) accumulation, and decreased ATP production) can all be associated with a neuronal Mn-deficiency via "starvation" of Mn-dependent enzymes. Furthermore, recent studies have shown that several Mn-responsive proteins and cell signaling pathways (i.e. AKT/mTOR, ATM/p53) are also impaired in HD. Interestingly, numerous studies have therapeutically targeted these exact same pathways in HD models with great success. This review will primarily discuss Mn-dependent enzymes and Mn-responsive proteins and how a neuronal Mn-deficiency may contribute to HD phenotypes and symptoms.

**Key Words:** *manganese, Huntington's disease, HTT, PI3K, AKT, mTOR, ATM, p53, cell signaling*

## Introduction

**Huntington's disease (HD)** is an age-progressive neurodegenerative disease causing mood and behavioral changes, but most noticeably a phenomenon known as chorea — uncontrolled, hyperkinetic movements. The root cause of these symptoms is death of GABAergic medium spiny neurons (MSNs) in the basal ganglia, particularly the striatum. However, the exact cause of this cell death remains unknown but is most likely multi-faceted. There is no known cure for HD and most treatments are aimed at relieving associated symptoms of the disease. Most notably, tetrabenazine is the sole approved drug to treat chorea. Minocycline, trehalose, creatine, coenzyme Q10, and HDAC inhibitors have been used to target huntingtin protein (HTT) proteolysis and aggregation, as well as mitochondrial and transcriptional dysfunction<sup>1</sup>. The age of onset (AO) of the disease is inversely correlated to the number of CAG (glutamine) repeats within the HTT gene — 35-40 repeats being the pathological threshold for the disease. Larger repeats have been associated with juvenile, early-onset HD<sup>2</sup>. The mean onset is 35-44 years old

and median survival is 15-18 years after onset, with symptoms getting progressively worse over time<sup>3</sup>. Huntington's patients experience dementia, mood changes, and metabolic (mitochondrial and glucose) dysfunction and thus, causes of death include pneumonia, suicide, cardiac failure, and accidents due to involuntary movements<sup>4-10</sup>. HTT codes for the huntingtin protein, and though its entire function is still a mystery, it is known to be critical for neuronal and synaptic development as well as embryogenesis<sup>11,12</sup>. HTT activates BDNF, facilitates autophagy, and functions broadly as a scaffolding protein<sup>13-19</sup>. The expanded protein (often referred to as mutant HTT or mHTT) has been implicated in disease progression, but the exact nature of the relationship is still unknown. The expanded CAG repeats cause mutant HTT to be more prone to forming aggregates, which have been suggested to be causal in the disease<sup>7,20</sup>. This is a pathophysiological feature shared between almost all CNS neurodegenerative diseases. While HD affects a small percentage of people (6:100,000), it is autosomal dominant and nearly 100% penetrant, making it

capable of crippling whole generations of families<sup>7</sup>.

HD is monogenic and monoallelic — that is, if one has more than 40 CAG repeats in either of their HTT alleles (normally ~20 copies), they will develop the disease at some point in their life, barring unnatural death<sup>7</sup>. However, AO is highly variable. Between patients with the same repeat length, AO can vary several years, even decades. Even between monozygotic twins, AO can vary up to 7 years<sup>21</sup>. This suggests that other genetic modifiers or environmental factors (such as heavy metals) may exist and are capable of dictating the exact age

of onset. In fact, Wexler and colleagues estimated that over 50% of the variability in age of onset is caused by environmental factors<sup>22</sup>. Lastly, the fact that mutant HTT protein is produced since embryogenesis but HD symptoms usually don't present until adulthood supports the theory that an age-related, environmental factor may contribute to the disease. While few environmental factors have been identified thus far, evidence supports a pathogenic role for manganese in Huntington's disease. This review is aimed at merging the existing connections between HD and Mn and presenting the evidence for a pathogenic role for Mn in HD.

## Metals and Neurodegenerative Disease

Dysregulation of metals, particularly heavy metals including iron, copper, manganese (Mn), and zinc have been commonly associated with disease states including neurodegenerative disease. All of these metals serve unique and indispensable functions in the healthy population, however most are toxic in excess. Iron, in particular, is essential for hematopoiesis — the creation of blood cells and other associated cell types. Furthermore, iron is a critical factor for several enzymes, including three of the four complexes of the mitochondrial electron transport chain<sup>39,40</sup>. Copper plays an essential role in oxidase and oxygenase activities and controlling levels of reactive oxygen species. Between 3-10% of all proteins in our body depend on zinc to fold correctly and subsequently adopt a correct conformational change<sup>35,36</sup>. Iron toxicity was first associated with Parkinson's disease (PD) in 1988 when autopsied patient brains revealed small black deposits of iron in the substantia nigra pars compacta — the region of the brain that degenerates in PD<sup>23,24</sup>. Prior to this, improper iron homeostasis had also been implicated in Alzheimer's disease (AD)<sup>25-29</sup>. Copper toxicity is directly linked to Wilson's disease, which often presents with cognitive impairment and parkinsonian bradykinesia<sup>30-32</sup>. Copper toxicity has also been associated with other neurological disorders including Menkes disease, Huppke-Brenl syndrome, Parkinson's and Alzheimer's diseases<sup>31,33</sup>. Similarly, zinc toxicity has been implicated in Alzheimer's disease, particularly through interactions with amyloid precursor protein (APP), as well as Parkinson's

disease and amyotrophic lateral sclerosis (ALS)<sup>34-38</sup>. Mn toxicity, commonly observed in miners and welders, has been thought to be causal in a Parkinsonian-like condition known as manganism<sup>41,42</sup>. However, only recently have defects in Mn homeostasis been implicated in HD pathophysiology<sup>43-46</sup>.

Interestingly, zinc and Mn concentrations are often inversely correlated with iron and copper concentrations, particularly in the basal ganglia, although they are thought to share the same transporters<sup>47,48</sup>. This unique relationship may relate to some of the heavy metal associated pathophysiology of HD, which will be discussed subsequently. Mn is critical for proper function of a diverse set of proteins and enzymes including manganese superoxide dismutase (MnSOD), arginase, glutamate synthase, and MRE-11. However, little has been elucidated on the transport of Mn in the brain, thus making Mn research somewhat of a "black box." While studies have discovered a set of diverse, shared transporters which are proposed to transport Mn such as transferrin, DMT1, and ferroportin, the exact roles, expression levels, and tissue/compartamental specificity are far from known<sup>49</sup>. Tools and techniques to measure and study Mn uptake and homeostasis do exist but are limited: cellular Fura-2 manganese extraction assay (CFMEA), graphite furnace atomic absorption spectrometry<sup>50</sup> and Inductively Coupled Plasma Mass Spectrometry (ICP-MS)<sup>51,52,53</sup>. Recently, a high-throughput chemical screen discovered a set of chemical inhibitors which could increase or decrease Mn-uptake in HD knock-in striatal neuroprogenitors cells (STHdhQ7/Q7 and

STHdhQ111/Q111)<sup>54, 55</sup>. These inhibitors could serve as a dynamic tool to study Mn homeostasis.

While few direct connections have been elucidated between HD and Mn, a significant body of evidence strongly supports two interconnected observations:

1) *Mn homeostasis is largely dictated by the homeostasis of other metals (i.e. iron and cop-*

*per), which have been repeatedly associated with neurodegenerative diseases including HD.*

2) *HD cell signaling and enzymatic phenotypes are almost uniformly consistent with neuronal Mn deficiency — both of which present with inadequate function of biologically indispensable, Mn-dependent, proteins and enzymes.*

## Mn and Huntington's Disease

Several correlations between Mn and HD have been recently elucidated, strengthening the case for a Mn-HD interaction. First, Mn levels are highest in the basal ganglia, particularly in the globus pallidus and caudate putamen (two brain regions that exhibit the highest degree of disease-related degeneration) and are found to increase with age<sup>56, 57</sup>. Secondly, Mn concentrations are often reduced in HD brain autopsies while iron and copper levels are high — the latter of which has been implicated in HTT aggregation<sup>58</sup>. This inverse relationship was observed in HD mouse cells by saturating iron transporters (i.e. Transferrin, DMT1) which partially blocked Mn uptake<sup>44</sup>. Furthermore, a mutation in ferritin, which functions as the primary

iron store, causes a condition where iron accumulates in the brain known as neuroferritinopathy. This condition often mirrors HD so closely that patients are often misdiagnosed with HD<sup>59-61</sup>. At a molecular level, the third and perhaps most convincing line of evidence lies in the fact that HD cell and in vivo models consistently present with decreased Mn-uptake, indicative of brain Mn deficiency<sup>43, 45, 62</sup>. Recently, this Mn-uptake defect has been found to be causal in an ATM-p53 cell signaling defect in mouse striatal StdhQ7/Q7 and StdhQ111/Q111 cells as well as patient-derived human induced pluripotent stem cells (hiPSCs) differentiated into striatal-like neuroprogenitors, which exhibited the first HD-relevant pathogenic role for Mn uptake<sup>43</sup>.

## Mn-Dependent Enzymes and Pathways

Dysfunctional metal homeostasis contributes to many functional defects including mitochondrial dysfunction, excitotoxicity, and oxidative stress, which are observed in numerous, if not all, neurodegenerative diseases. Specifically in HD, there is a striking overlap in known pathological phenotypes and known Mn-responsive or dependent pathways and enzymes, supporting the hypothesis that dysregulation of Mn homeostasis may contribute to HD pathophysiology. Further, the nature of these HD phenotypes is near uniformly consistent with neuronal Mn deficiency. Although there are no known

neurological conditions due to brain Mn-deficiency, in HD several critical Mn-dependent enzymes possess reduced activity and manifest as lesser-known, but repeatedly observed symptoms and phenotypes. Furthermore, the observed cell signaling defects in p53/ATM, PI3K/AKT, and mTOR pathways in HD models are completely consistent with brain Mn-deficiency (see below). This suggests that Mn dysregulation may act upstream of HD disease pathology (i.e. enzymatic and cell signaling defects), supporting the therapeutic potential of modulating Mn homeostasis.

## Mn-Dependent Enzymes and Huntington's Disease

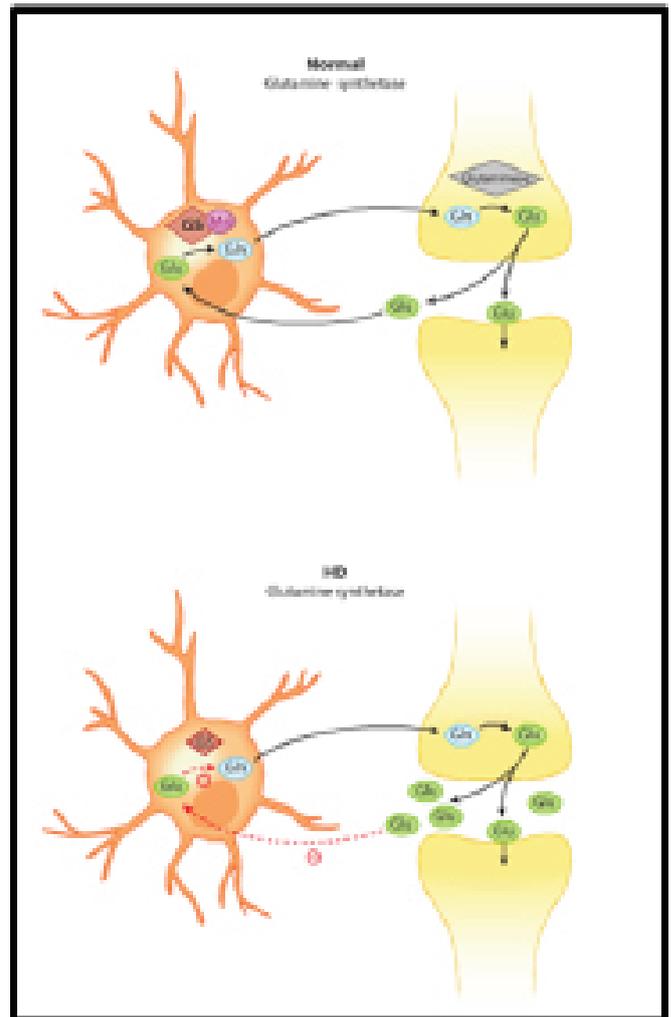
Dysregulation of Mn has been associated with impaired glutamate-glutamine homeostasis in the brain. **Glutamate synthetase** is a manganese-dependent enzyme that is found primarily in astrocytes. In fact, Mn

bound to glutamine synthetase represents about 80% of total cerebral manganese<sup>63-67</sup>. Glutamate synthetase functions to convert the neurotransmitter glutamate into glutamine<sup>63, 64</sup>. Glutamine can then be readily

readily taken up by nearby neurons and converted back into glutamate. This process acts to buffer glutamate, protecting surrounding neurons against excitotoxicity. In HD, it has been proposed that this process goes awry and brain Mn-deficiency leads to impaired activity of glutamate synthetase in astrocytes, impairing further glutamate uptake. This causes a build-up of glutamate around synapses, increasing proclivity for seizures and excitotoxicity (**Figure 1**). These are two common phenotypes associated with HD; the latter primarily in juvenile onset cases<sup>68-72, 49</sup>. It has been suggested that either Mn neurotoxicity or deficiency can cause convulsions and some forms of epilepsy<sup>73 74-76 77</sup>. Taken together, this offers a potential link between a manganese-dependent enzymes and HD phenotypes.

**Arginase** is a manganese-dependent enzyme required for production of urea and removal of toxic ammonia. Both isozymes of arginase (ARG1, ARG2) have near identical functions and have been found in the brain and other tissues, with the highest levels of ARG2 found in the neocortex, corpus collosum, putamen, and ventral striatum<sup>78</sup>. While its function in the brain hasn't been fully elucidated, studies have suggested that it plays a neuroprotective role. ARG1 functions to deplete arginine, inhibit nitric oxide synthesis and promote neuronal survival<sup>79-82</sup>. Arginase also plays a role in the neural immune response by activating microglial pathways<sup>83</sup>. Interestingly, increased ammonia and reduced arginase activity occur in both HD mouse models and human patients, suggesting improper activation of arginase by Mn and presenting another avenue of pathogenicity for Mn<sup>44, 84, 85</sup>.

**Manganese superoxide dismutase (MnSOD/SOD2)** is an antioxidant mitochondrial metalloenzyme, which functions to convert superoxide ions into H<sub>2</sub>O<sub>2</sub> and oxygen, protecting the cell from reactive oxygen species production and death. MnSOD binds with Mn, which is necessary for its complete activation. Thus, it is not surprising that Mn supplementation can increase the activity of MnSOD<sup>86, 66</sup>. Manganese deficiency decreases MnSOD activity and lipid peroxidation in mitochondria, a phenotype also seen in HD patients<sup>87-89</sup>. This reduction in MnSOD activity is suggested to cause mitochon



**Figure 1:** Taken from *Horning et al, 2015*. The role of Mn-dependent glutamine synthetase (GS) in normal and HD conditions. In normal conditions, GS functions to convert glutamate (Glu) to glutamine (Gln), clearing excess glutamate from the synapse. In HD, it is proposed that decreased Mn-uptake causes reduced activity of GS, an accumulation of Glu at the synapse, and subsequent excitotoxicity and potentially seizures.

drial toxicity under conditions of manganese deficiency which corresponds with impaired energy production (reduced ATP levels) in HD models and patients<sup>6, 90-95</sup>. P53 is also known to inhibit expression of MnSOD, consistent with increased p53 levels and oxidative stress in HD<sup>96</sup>. Another mitochondrial enzyme, **pyruvate carboxylase** is also Mn-dependent and an essential member of the tricarboxylic acid (TCA) cycle — converting pyruvate into oxaloacetate. Loss of its activity results in in-

observed in HD models and patients<sup>74, 75, 97-101</sup>.

The HTT gene expands (gains more CAG repeats) both between generations and throughout lifetimes as expansions fail to be corrected by cellular repair mechanisms. Because of this, HD has been associated with genomic instability. However, the very nature of these long GC rich CAG repeats makes it more susceptible for errors during replication and thus, CAG expansion might not truly be indicative of genomic instability. Somewhat counter-intuitively, risk for HD and many other neurodegenerative diseases is often inversely related to risk for cancer, which is commonly associated with genomic instability. In fact, one study shows that HD patients have a 6 times decreased chance of cancer than their own siblings<sup>102</sup>. Furthermore, many DNA repair enzymes are Mn-dependent or

responsive including **p53, ATM, MRE-11, and translesion polymerases**. MRE-11 is an obligate member of the MRN complex which activates ATM (ataxia telangiectasia mutated) — which itself is Mn-responsive — in the presence of DNA damage<sup>43, 103-105</sup>. MRE-11 has a di-manganese pocket, which is necessary for its exonuclease activity during DNA double-strand break repair. Translesion polymerases are Mn-responsive polymerases responsible for bypassing DNA lesions to perpetuate DNA replication and thus, by nature, have high error rates<sup>106-108</sup>. A Mn-deficiency in HD may contribute to reduced activity of these error-prone polymerases while increasing activity of MRE-11, p53, and ATM, reducing cancer risk. Taken together, Mn may act upstream of the observed altered DNA repair, genomic instability, and decreased propensity for cancer found in HD patients and models<sup>109</sup>.

## P53/ATM and Mn in Huntington's Disease

*TP53*, a tumor suppressor gene, has been consistently linked to HD for decades. P53 has been implicated in dozens of cell signaling pathways but is probably most well-known for activation of DNA repair, cell cycle arrest, and apoptosis — all of which have been associated with HD. Interestingly, Ataxia telangiectasia mutated (ATM), a Mn-dependent kinase, is responsible for phosphorylating several key DNA damage proteins including p53, CHK2, and H2AX following DNA double strand breaks. Both p53 and ATM have been reported to be upregulated in HD, and inhibition of those proteins rescues HD phenotypes<sup>110, 111</sup>. Genetic variation in TP53 has been proposed to account for ~13% of the CAG-independent AO variability in HD112. However, p53 is suggested to regulate HTT expression<sup>113</sup> and mutant HTT can dysregulate transcriptional regulation via interaction with P53 and CREB<sup>114-116</sup>. P53 accumulates in HD patients, and primates exposed to Mn show increased levels of downstream p53 transcripts<sup>117</sup>. A recent paper showed that after an exogenous, sub-toxic manganese exposure, HD cells exhibited the largest cell signaling impairment in phosphorylation of p53 (of the 18 protein phosphorylation events tested). This study concluded that this defect was due specifically to inadequate activation of ATM by Mn<sup>43</sup>. The defect was then rescued using KB-R7943 (an inhibitor of NCX1/3 sodium-calcium uniporters), which corrected the Mn-uptake de-

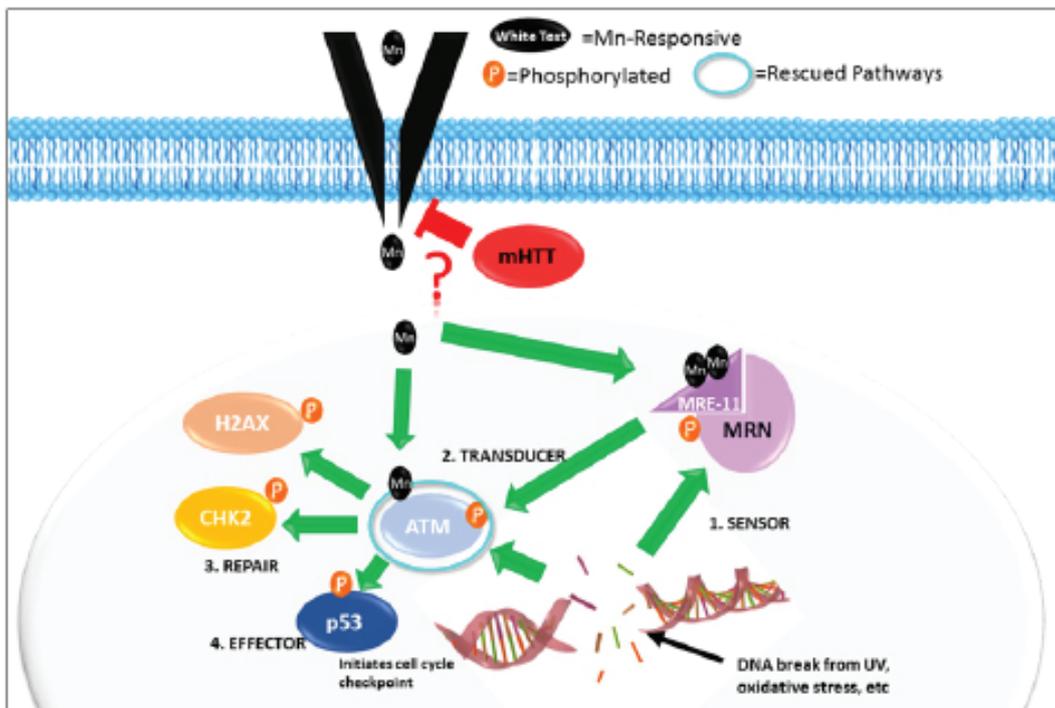
fect and thus the responsive cell signaling defects. This implicates both p53 and ATM in HD pathogenesis via a Mn homeostasis defect. Contrary to these findings, Lu and colleagues discovered that ATM is hyperactive in both HD models and patients, (without an exogenous Mn exposure) in HD and inhibition (small molecule inhibitors, knockdown, or heterozygous knock-out) of ATM in various HD models could rescue HTT associated toxicity and remedy some motor and behavioral deficits<sup>111</sup>. There are several reasons for these discrepancies. Most notably, Lu and colleagues examined basal levels of ATM while Tidball et al. examined the pathway after a sub-toxic Mn exposure, which is less biologically-relevant. Thus, both papers examine the pathways under very different conditions. Also, Tidball et al. established this relationship primarily in cell culture while Yang et al., used drosophila, mouse, and cell-based models. However, both groups use patient-derived hiPSC models. The nature of the interaction between HTT and ATM remains to be seen, as neither group has established an exact role for this interaction. **While decreased ATM activation is consistent with decreased Mn levels and MRE-11 activity, hyperactivation is consistent with patient samples and HD models, which have shown increased levels of downstream p53. While these two studies may be somewhat at odds with each other, they irrespec-**

tively implicate a Mn-dependent enzyme and pathway (ATM and MRE-

## PI3K/AKT pathway and Mn in Huntington's Disease

AKT is another well-studied, highly conserved kinase, and its upstream kinase and activators PI3K, IGF-1, and insulin have been more recently implicated in HD pathology. Insulin and insulin growth factor 1 (IGF-1) activate PI3K, which leads to activation of AKT. Separately, phosphorylation via ATM is necessary for complete activation of AKT<sup>118</sup>. AKT then dictates a range of processes by stimulating cell growth, proliferation, and survival<sup>119</sup>. AKT interacts with a variety of other proteins including MDM2, which can inhibit p53, Pras40 and TSC1/2, which can inhibit mTOR, Ataxin-1, and GSK-3 $\beta$ . Multiple reports have indicated that HD models, HD patients, and manganese-deficient models both exhibit decreased IGF-1 and insulin levels and AKT activation<sup>120</sup>. Moreover, AKT also phosphorylates HTT and reduces aggregation and eliminates associated toxicity<sup>121, 122</sup>. However, it has also been shown that Mn exposure can significantly increase both IGF-1 levels and phos-

phorylation of AKT in various HD models<sup>45, 122-125</sup>. Furthermore, activation of AKT, particularly via phosphorylation of Ser473, has been associated with neuroprotection<sup>126</sup>. Treatment with the upstream growth factor IGF or insulin and can rescue AKT phosphorylation in both HD cells and mouse models, increasing HTT phosphorylation and decreasing mHTT toxicity<sup>122, 123, 127, 128</sup>. This also improves mitochondrial function and metabolism in HD mouse primary cultures and lymphocyte cultures<sup>124, 128</sup>. It isn't surprising given these defects with insulin and IGF-1 signaling that HD patients are at an increased risk for hyperglycemia and type II diabetes<sup>129, 130</sup>. Similarly, Mn-deficiency and toxicity has been shown to impair glucose and insulin metabolism<sup>120, 131, 164</sup>. These studies suggest the possibility that Mn treatments could ameliorate mHTT toxicity and metabolic dysfunction — two widely observed HD phenotypes.



**Figure 2: Mn and ATM/p53 pathways in HD.** The canonical role for this pathway is in the presence of DNA double strand break which activates the MRN complex and ATM. However, Mn can also directly activate ATM and MRE-11 and also indirectly activate p53 through ATM. Both MRE-11 (sensor) and ATM (transducer) can be activated by Mn. After activation, ATM can activate p53, H2AX, and CHK2 (effector and repair proteins) to initiate G1/S cell cycle checkpoint recruit the appropriate proteins. Mutant HTT (mHTT) decreases Mn-uptake through an unknown mechanism.

## mTOR and Mn in Huntington's Disease

The mTOR pathway is the most recently associated signaling pathway with HD. Downstream of PI3K and AKT is the mechanistic target of rapamycin (mTOR), a kinase responsible for regulation of cell growth and metabolism. It forms two complexes: mTORC1 and mTORC2. mTORC1 is directly responsible for activating downstream targets — primarily S6 Kinase, S6, and an array of transcription factors which bind to DNA initiating transcription and translation. This causes an upregulation in protein synthesis and cell growth. However, until recently, the potential for an HD-mTOR connection was not fully acknowledged. Two groups published studies portraying seemingly polar opposite roles for mTOR in HD<sup>132, 133</sup>. The first paper by Subramanian and colleagues provides evidence that mutant HTT can upregulate mTOR signaling, particularly through amino acid accumulation and mediation. Furthermore, deleting TSC1, a protein that inhibits mTOR, exacerbates HD related behavioral deficits and accelerates death<sup>133, 134</sup>. Additionally, they could abrogate mTOR activity by inhibiting PI3K or knocking down Rheb, two upstream activators of mTOR (Figure 3). This corresponds with earlier reports that similar mTOR disruption caused neurodegeneration<sup>135, 136</sup> and that mTOR inhibition via rapamycin can block anxiety and depressive HD-like behaviors<sup>137</sup>. The second paper was published a few months later when Thompson, Davidson and colleagues showed that mTOR is downregulated in various models as well as HD patients, and that activation of mTOR could not only improve striatal

cell function, but improve motor phenotypes in an in vivo mouse model<sup>138</sup>. Thus far, the evidence and general consensus within the HD community seems to agree with the conclusion of the latter paper: mTOR signaling is impaired in HD. Subramanian never directly shows that inhibiting mTOR ameliorates HD phenotypes, only that activating mTOR enhanced disease progression. Also, Thompson and colleagues draw multiple parallels to patient samples and show that activating mTOR can directly rescue HD-phenotypes both in cells and mouse models. Similarly to p53/ATM and AKT pathways, the mTOR pathway is also responsive to Mn<sup>139</sup>, suggesting the possibility that Mn supplementation could rescue mTOR signaling in HD models similar to the findings Thompson and colleagues. Given that the pathway exists downstream of the well-characterized and Mn-responsive PI3K/AKT pathways, it seems likely that mTOR and downstream targets are also Mn-responsive and defective in HD. Further studies will need to answer the existing discrepancy on mTOR's role in HD — either neuroprotective or pathological. Furthermore, additional studies are needed to examine the responsiveness of mTOR to Mn and whether this relationship contributes to the disease.

**These relationships between Mn homeostasis and HD pathophysiology not only add validity to a Mn-HD interaction but may provide potentially novel targets for future preventative and neuroprotective “manganese-centric” therapies.**

## Conclusion and Future Directions

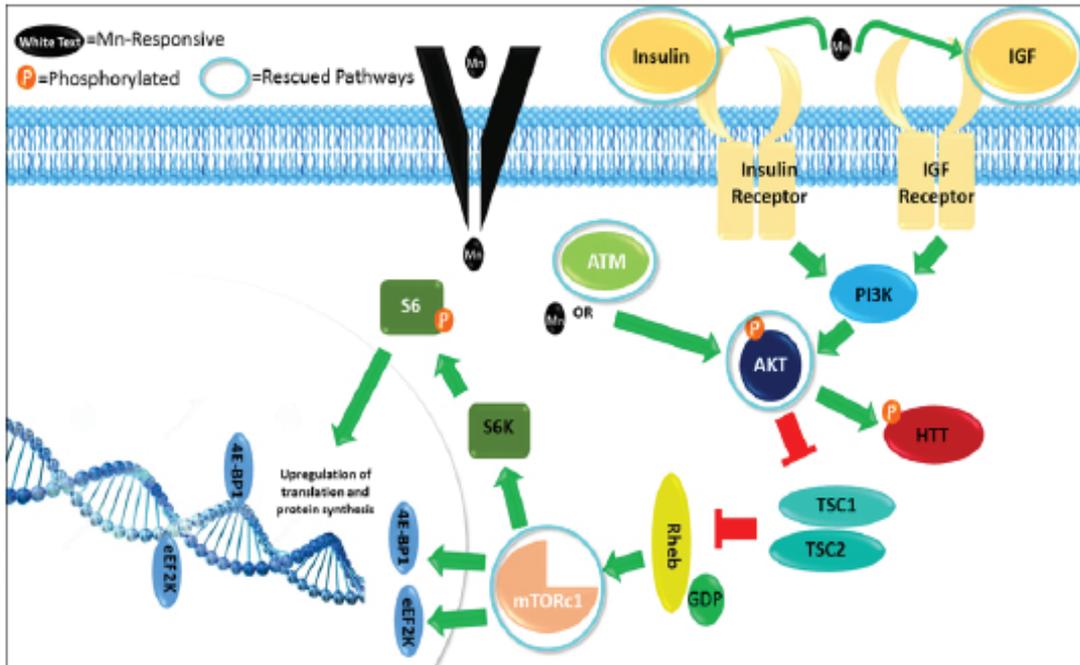
Although evidence for a pathogenic role for Mn in HD has been accumulating, there is still a dearth of knowledge concerning its exact role in disease age of onset, progression, and symptoms. Understanding how Mn is transported and regulated in the brain is of utmost importance. We still know very little about the homeostatic control of Mn in the brain. An aforementioned high-throughput screen, validating several inhibitors that can change Mn-uptake in neural cells may offer insight into the existing “black box” of neuronal Mn transport. Some studies have shown that HD patient brains exhibit decreased Mn levels in the brain,

but the data is inconsistent. While a Mn-deficiency has been shown in striatal models of HD, few studies have examined Mn-levels in other cell types in the brain (dopaminergic, cortical, astrocytes). Furthermore, these studies examined these effects in neuroprogenitors only, not mature neurons, begging the following question: Are these defects only present in neuroprogenitors or do they continue into fully functioning neurons, particularly mature medium spiny neurons (MSN's) — the main cell type lost in HD? Other pertinent questions in the field include: what is the exact relationship between Mn and the HTT protein? And how does

mutant HTT lead to a Mn deficiency? Studies up to this point have been correlative observations between mutant cells and a Mn transport defect but have not offered a true connection between the two. Foremost, it must be discerned whether

**1) Mutant HTT directly causes a neuronal Mn**

**deficiency and dyshomeostasis condition with a potential role in HD pathology, 2) Mutant HTT causes extensive neurotoxicity through HD pathology, which in turn leads to Mn handling defects parallel to disease processes, or 3) some intermediate role between the two.**



**Figure 3; Mn and AKT/mTOR pathways in HD.** Mn can increase Insulin and IGF levels, which activate PI3K, and AKT. ATM and Mn can also directly activate AKT. AKT can phosphorylate HTT which can abrogate mHTT-associated toxicity. Downstream, mTOR and S6K can also be activated by Mn. This leads to increased translation, protein synthesis, and cell growth through a variety of transcription factors. AKT, IGF, insulin, and mTOR pathways have been targeted to rescue HD- phenotypes in various models (circled). Mutant HTT (mHTT) decreases Mn-uptake through an unknown mechanism.

Enzyme	Function	Mn interaction	Associated HD-phenotype	Source
Glutamine synthetase	Converts glutamate to glutamine	Binds manganese	Increased excitotoxicity Increased propensity for seizures (juvenile-onset)	(Carter 1982)(Wedler and Denman 1983, 2014)
Arginase	Production of urea and removal of ammonia	Binds 3 Mn(II) ions	Increased ammonia and reduced arginase activity	(Chiang, Chen et al. 2007) (Chiang, Chen et al. 2009)
MnSOD/SOD2	Antioxidant; dismutates superoxide ions	Necessary cofactor for activity	Increased oxidative stress and reduced mitochondrial function	Zidenberg-Cherr, Keen et al. 1983)
Pyruvate Decarboxylase	Converts pyruvate into oxaloacetate in TCA cycle	Needs to bind Mn for activity	Impaired ATP production and increased lactate production	(Lee, Ivanova et al. 2007)(Mochel, Charles et al. 2007)
MRE-11	Necessary for NHEJ DNA repair after DNA double-strand break	Binds two Mn(II) ions responsible for exonuclease activity	Reduced ATM activity*	(Tidball, Bryan et al. 2015)

**Table 1: Function, interaction with Mn, and associated HD phenotypes for Mn-dependent enzymes discussed.**  
\*opposite phenotype was observed by Yang et al. 2015

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## **Further Information**

<https://www.mc.vanderbilt.edu/root/vumc.php?site=neurology&doc=26214>

# The Role of Audiovisual Integration in Auditory Re/instatement Through a Cochlear Implant

*Iliza M. Butera*

## Abstract

Cochlear implants (CIs) allow those with profound hearing loss to experience sound, some of them for the first time. This highly successful neuroprosthetic device can drastically improve speech comprehension for some individuals; however, postoperative speech proficiency remains highly variable and difficult to predict. Although visual orofacial articulations play a crucial role in verbal communication both before and after cochlear implantation, clinical measures assessing implant candidacy and monitoring postoperative performance are currently limited to auditory-only speech measures. As a result, current assessments may be providing a partial picture of aural rehabilitation with a CI. The aim of this review is to highlight recent work investigating the role of audiovisual integration in auditory re/instatement. This knowledge is essential for our understanding of proficiency with a CI and, most importantly, for how users can best utilize all sensory information to enhance intelligibility and improve quality of life.

## Introduction

Cochlear implantation is an effective surgical intervention to either restore progressive hearing loss or, for the congenitally deaf, establish it for the first time. The technology behind this auditory re/habilitation is based on an external processor first downsampling sound into coarse frequency bands. These electrical signals are then transmitted to an implanted electrode where surviving spiral ganglion cells are stimulated in a tonotopic manner<sup>1</sup>. This spectral parcellation, albeit crude, mimics normal frequency filtering in the cochlea by localizing high and low frequencies respectively to the base and apex. Furthermore, amplitude envelope cues that are crucial for speech intelligibility are preserved through channel-specific electrical current modulation<sup>2</sup>. Though lacking fine spectral detail, these inputs roughly encoding both frequency and amplitude are delivered to the auditory periphery with high temporal resolution, purportedly within a range similar to acoustic hearing<sup>3</sup>.

Modern intracochlear arrays can contain up to 20 electrode contacts, yet the fidelity of frequency encoding is limited to approximately 7 or fewer channels<sup>4</sup>. This limitation is due to current spreading between neighboring electrodes via

the surrounding perilymph. In addition to this compromising technical limitation, the nervous systems of CI candidates have varying degrees of central and peripheral atrophy and/or aberrant remodeling as a result of sensory deprivation. Thus, in CI users a primitive representation of sound is delivered to as few as 15% of the typical number of spiral ganglia<sup>5</sup> with subsequent auditory processing by naïve primary and associative cortical areas. Considering the presumed diversity of these underlying anatomical features and the interindividual differences in severity of hearing loss, onset and duration of auditory deprivation, as well as mode of communication and language proficiency, it is perhaps unsurprising that speech outcome measures for CI recipients are both highly variable and difficult to predict<sup>6,7</sup>.

Degraded auditory input is common to all CI processors and, like hearing loss, prompts attentional focusing on complementary sensory modalities. Speech processing is typically an audiovisual experience where coincident orofacial articulations can considerably boost intelligibility over uni-sensory auditory thresholds<sup>8,9</sup>. This is also true for typical listeners who can benefit from visual

speech cues to communicate in otherwise unintelligible auditory signal-to-noise ratios. Thus, when faced with impaired auditory inputs, either acoustically or electrically, the incorporation of visual cues is an intuitive and effective compensatory strategy. A growing body of literature is focused on defining the role of vision in speech recovery in terms of behavioral and neuroana-

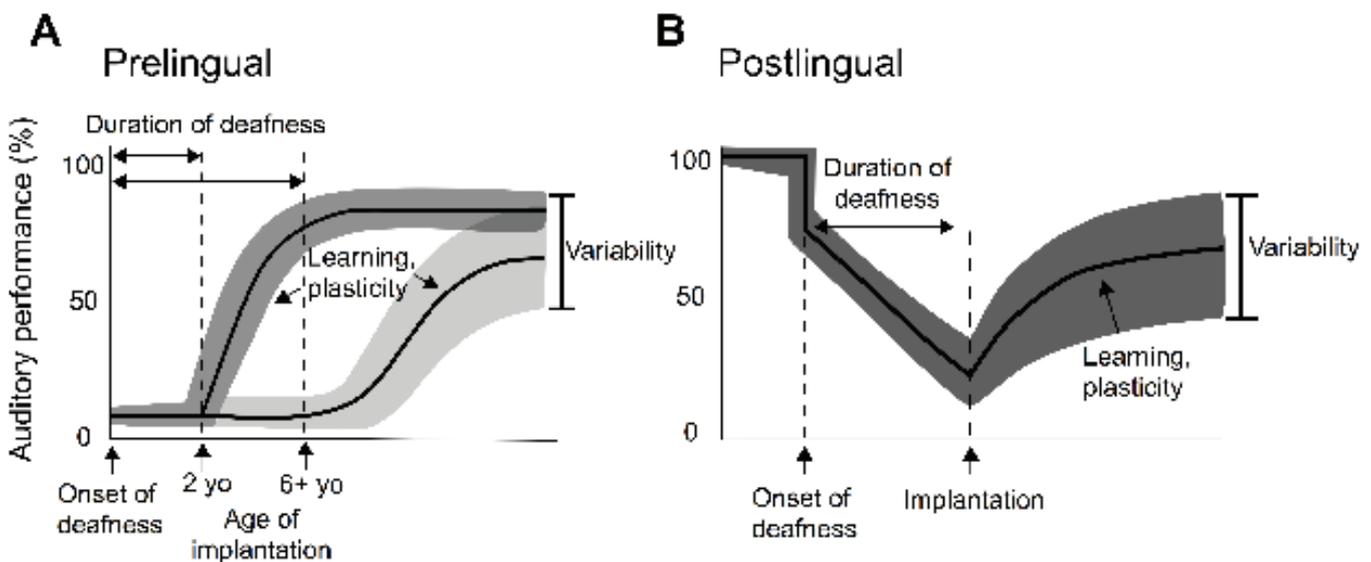
tomical markers of audiovisual fusion as well as unique visually-driven cortical activation in the auditory-deprived brain<sup>10-12</sup>. Here, I review the known predictors for aural recovery through a CI and recent work describing neuroplastic changes at behavioral and structural levels in auditory and visual cortices in order to address the role of audiovisual integration in auditory re/habilitation.

## Clinical Outcome Measures and Known Predictors of Their Variability

Pure-tone hearing thresholds determine adult CI candidacy, and severe-to-profound hearing loss is defined by thresholds in the range of 70 to 90 dBHL. Post-operative outcome measures of speech understanding include open-set monosyllabic word recognition<sup>13</sup>, sentence recognition in quiet<sup>14</sup>, and/or sentence recognition in noise (i.e. with 4 talker speech-like babble)<sup>15</sup>. The typical trajectory for improvements in these speech measures is characterized by a steep increase within the first few months post-activation where, for example, the percent of disyllabic words correctly identified averages 10% pre-operatively, 47% after several month's experience, and 81% at 1 year, followed by a stable plateau in performance<sup>16</sup>. Two clear driving forces for variability in such speech intelligibility measures are: 1) the duration of deafness

and 2) the age of implantation, particularly in regards to sensitive periods for language acquisition. Severe-to-profound hearing loss identified before age 2 is generally considered to be prelingual in onset (Fig. 1A), as opposed to sudden or gradual onset hearing loss identified after language acquisition later in childhood or adulthood (Fig. 1B).

Across age ranges, there are a variety of reports indicating a negative correlation between duration of deafness and speech understanding scores<sup>6,17-21</sup>. A similar physiological correlation has also been described with the duration of deafness and metabolic auditory cortical activation via PET imaging, suggesting that greater neural recruitment at the level of the primary auditory cortex (A1) is associated with shorter periods of auditory deprivation<sup>22</sup>.



**Figure 1.** Schematic of cochlear implantation after (A) prelingual or (B) postlingual onsets of profound hearing loss resulting in distinct learning trajectories and predictors of post-implant auditory performance. (Adapted in part from REF: 6,23)

In regard to both congenital and early postnatal onset of deafness, FDA approval for CI surgery extends to infants as young as 12 months old. In such cases of prelingual hearing loss, auditory stimulation initiated during heightened periods of plasticity can result in very successful outcomes. For example, cochlear implantation before 3.5 years of age is associated with a rapid establishment of near-normal cortical auditory evoked potentials<sup>24</sup> as well as downstream benefits in language development when compared with peers implanted two years later<sup>25</sup>.

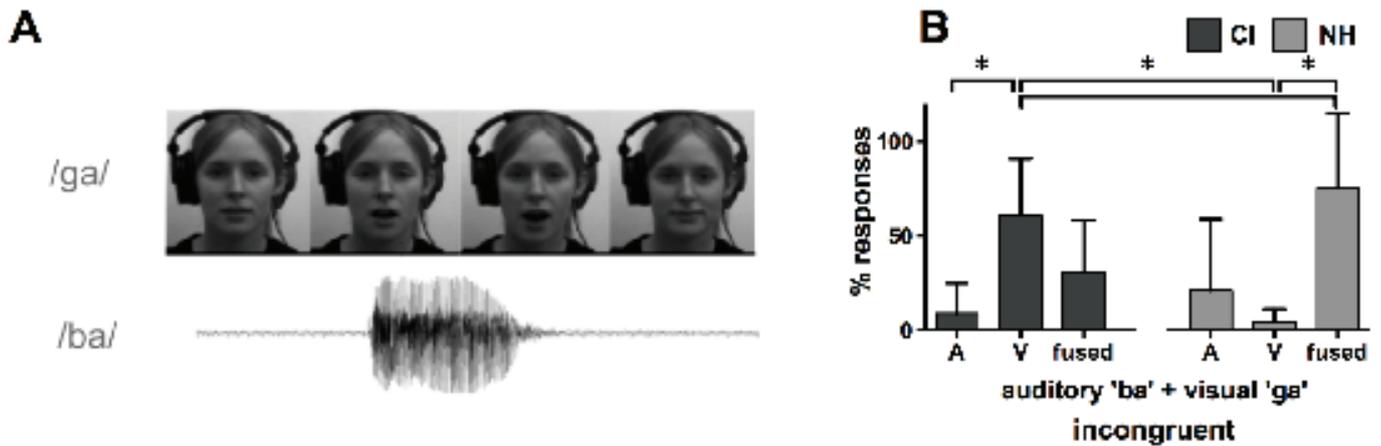
During these sensitive periods, extensive changes in anatomical connectivity occur in order for humans to adapt to relevant sensory environments and stabilize these established networks in the mature brain<sup>26</sup>. Neuroplasticity is a feature of both development and learning that is also well known

to impact sensory circuits as a result of sensory deprivation—particularly during early postnatal life<sup>27</sup>. Studies in animal models of auditory deprivation have greatly informed the timing and duration of critical periods for auditory development of subcortical and cortical anatomical tracts<sup>28</sup>, tonotopicity<sup>29</sup>, binaural hearing<sup>30</sup>, audiovisual integration<sup>31</sup>, and normal firing rates in the auditory cortex<sup>32</sup> as well as Local Field Potentials (LFP)<sup>28</sup>. Clinical studies indicating variable trajectories of learning and speech acquisition when cochlear implantation occurs after critical periods in auditory and language development, further highlight the need for early auditory stimulation for successful habilitation<sup>33,34</sup> (Fig. 1). Indeed, the same considerations are important for surgical interventions for blindness, such that stimulation of peripheral sensory receptors doesn't necessarily ensure functional utility of these signals in a sensory-deprived brain<sup>35</sup>.

## Benefits of Multisensory Integration in CI Users

Increased saliency<sup>36</sup>, decreased detection thresholds<sup>37,38</sup>, and reduced reaction times<sup>39</sup> are all indicative of the cooperative advantages of bimodal sensory information. Integration between sensory modalities enhances perception in either a sub or supra-additive manner depending on whether stimuli are complementary or contradictory<sup>40</sup>. Perceptual enhancement in the form of increased speech saliency has clear advantages for oral communication, particularly in noisy environments<sup>8</sup>. Given the enhanced ability to discern visual-only speech compared to normal-hearing controls, it has been suggested that CI users compensate for auditory deficits with enhanced visual proficiency, thereby achieving comparable audiovisual perception to controls<sup>16</sup>. Furthermore, early implantation (i.e. before age 4) has been shown to result in faster reaction times<sup>39</sup>, greater multisensory gain<sup>41,42</sup>, and higher speech recognition at multiple levels of phonetic processing<sup>18</sup>.

Although the average postlingually deafened adult CI user can typically achieve 80-90% accuracy with sentence-level tests in quiet<sup>43</sup>, hearing in noise is considerably more difficult and variable between users<sup>44</sup>. The presence of noise decreases saliency such that multisensory gain is increased via the principle of inverse effectiveness. Additional predictors of multisensory gain are whether sensory stimuli are coincident in time and space. When conflicting audiovisual speech content is aligned in both dimensions, crossmodal illusions can be perceived<sup>45</sup>. First described in the late 1970s, the McGurk illusion results from the integration of an extracted sound file of the syllable “ba” paired with the visual articulation of “ga” eliciting the percept of a third syllable such as “da” or “tha”<sup>46</sup>. This illusory task has been used as a proxy for quantifying multisensory integration, and widely consistent results indicate a visual bias for CI populations<sup>47-49</sup>, with some reports of greater fusion or less visual bias in more proficient CI users<sup>50,51</sup>.



**Figure 2.** (A) Auditory and visual stimuli for the McGurk effect elicit the perception of a fused audiovisual token in normal hearing children. (B) Unpublished observations further corroborating data from several studies in CI users indicate a visual bias in CI children toward lipreading the visual stimulus and reporting the syllable “ga” over perceptual fusion. CI = cochlear implant (n = 18); NH = normal hearing (N = 18); A = auditory token; V = visual token; \* p<0.01; Fig. 2A adapted from REF: 52

In recent years the neural architecture responsible for modulating fused-token (i.e. “da” or “tha”) perception in the McGurk illusion has been localized to the multimodal processing area of the superior temporal sulcus (STS). Beauchamp and colleagues have correlated activity with interindividual variability in fusion<sup>53</sup>, as well as identified a causal role of STS activation during fusion via transcranial magnetic stimulation<sup>54</sup>. This auditory and audiovisual processing area is located just caudally to the primary auditory cortex and is responsive to both speech stimuli and objects, following the principle of inverse effectiveness for multisensory integration<sup>55</sup>. Interestingly, resting state medial to anterior STS connectivity has been correlated with language skills<sup>56</sup>, suggesting a mechanistic role between the transfer of behavioral activation via McGurk fusion and generaliza-

tion to other language measures. Indeed, higher activity of superior temporal regions, particularly in the left hemisphere, has been linked with greater phonological awareness and language processing through several imaging techniques<sup>57</sup>.

Furthermore, visual-only speechreading activates the left-posterior STS to a greater degree in deaf individuals than controls, and the fast onset of these functional differences—as little as 4 months after onset of deafness—suggests that latent multisensory areas are immediately activated with sensory deprivation<sup>58</sup>. This is in contrast to progressive remodeling via relatively slow synaptic plasticity<sup>59</sup>. Greater left lateralization of these networks in deaf individuals is suggestive of specialized language-processing networks in multimodal regions.

## Compensatory Language Development During Sensory Deprivation

Profound hearing loss requires compensatory strategies for communication by recruiting intact sensory modalities. Orofacial cues for speech reading, gestures for cued speech, and manual communication (i.e. sign language) can cause plastic changes as a result of visio-linguistic training. At the behavioral level, visual enhancements in individuals with profound hearing loss include:

1) enlarged peripheral visual fields<sup>60</sup>, 2) superior performance with peripheral visual tasks<sup>61,62</sup>, and 3) enhanced visual motion detection<sup>63</sup>. However, the size and responsivity of the primary visual cortex is seemingly unchanged according to functional magnetic imaging<sup>64</sup>. Furthermore, absolute visual sensitivity thresholds are also largely unchanged. Instead, behavioral adaptations

appear to be highly specialized to compensatory behavioral functions, particularly communicative adaptations. Better peripheral vision, for example, could be achieved through attentional training to communicate in the periphery through sign language while maintaining eye contact.

It is difficult to parse effects of sensory deprivation from those of behavioral training. For instance, auditory cortical activation by sign language could relate to visual colonization of the auditory cortex from sensory deprivation or, instead, could stem from a compensatory mechanism of visual language driving activity in areas that are typically auditory<sup>65,66</sup>. The inclusion of hearing signers helps to address this issue by identifying changes due to visuospatial communication itself (e.g. heightened peripheral attention) as opposed to changes that are unique to the auditory-deprived brain (e.g. visual moving dot activation of A1)<sup>67,68</sup>.

Compensatory language could also be a secondary source of such unique changes. For instance, feedback projections from area MT—an associative visual area largely responsible for motion detection—may interact with cortico-cortical projections between the primary visual and auditory cortices to account for generalizations of physiological and behavioral visual enhancements. That is, heightened motion detection in the right visual field in deaf individuals<sup>69</sup> may also correspond to a left lateralization of parietal-occipital networks interacting with dorsal language processing streams as well as associative visual areas like area MT.

In an investigation of visual-only speech reading performance, Suh et al. reported a negative relation-

ship between A1 latency and speechreading scores in postlingually deafened adults<sup>70</sup>. With increasing duration of deafness and postlingual onset, there were decreasing A1 latencies, perhaps indicative of more efficient speechreading networks developing with experience. This relationship, however, was absent in those with profound hearing loss with an onset prior to language acquisition. Thus, established speech networks may be required for perceptual enhancements through experience. Although not yet widely accepted, the establishment of language networks prior to profound hearing loss by any means necessary (i.e. orally or through sign language) has been proposed<sup>71,72</sup>.

Evidence for parallel auditory processing in dorsal and ventral streams has been a very recent finding<sup>73</sup> compared to similar mechanisms described in vision. In a unique study, Lazard and colleagues performed fMRI of deaf individuals prior to cochlear implantation<sup>74</sup>. Post-implantation behavioral assessments were then related to neuroimaging measures. (Unfortunately, post-operative fMRI is not possible due to magnetic artifacts from the implant and medical concerns of shifting electrodes, demagnetization of implants, and heating surrounding tissues.) These preoperative functional measures indicate dorsal phonological processing in more proficient CI users compared to more ventral processing in poor CI performers. These findings corroborate prior studies of resting metabolic activity in ventral temporal areas compared to dorsolateral prefrontal areas, which respectively correspond with non-proficient and proficient CI users<sup>7</sup>. Pre-operative neuroimaging represents a new avenue for better understanding post-operative variability in speech outcomes.

## Mechanisms of Crossmodal Plasticity

Direct projections have been described between tonotopically-organized regions of auditory cortex and peripheral visual field targets<sup>75</sup>. Although these have been known to exist during early postnatal life, only recently has their persistence into adulthood been explored in greater depth<sup>76</sup>. Such projections to early cortical processing areas suggest that auditory cues could facilitate detection in the peripheral field, even into adult-

hood. Thus, in the absence of auditory input, it is possible that these transient connections could be maintained at a higher proportion in the sensory-deprived brain than in normal maturation.

Evolutionary pressure to utilize inactive cortical processing areas, like those of the primary visual cortex in blind mole rats, causes alterations in long-range subcortical connectivity

(i.e. from the inferior colliculus to auditory as well as visual thalamus and cortex). Through these ectopic connections, auditory activation of typically-visual areas can enhance the processing of intact peripheral hearing organs<sup>77</sup>; nevertheless, cross-modal plasticity at this spatial scale is less common than local changes in cortical circuits<sup>78</sup>. For instance, in the deaf cat, Lomber and colleagues

have reported crossmodal plasticity in low-level sensory processing in the primary auditory cortex as well as higher associative cortices<sup>79</sup>. Furthermore, in these animals robust visual activation of typically-auditory areas like the field of the anterior ectosylvian sulcus (FAES) displays functional connectivity with visual orienting responses<sup>80</sup>.

## Perceptual Training

In a striking example of perceptual training, degraded frequency representations<sup>81</sup> and temporal processing<sup>82</sup> have been restored in adult rodent models well after critical periods, which—along with recent work exploring the reopening of critical periods in adults—suggests maintained plasticity in the mature mammalian brain<sup>83</sup>. Indeed, several studies have begun testing perceptual thresholds and their adaptability through audito-

ry training<sup>84</sup> in humans with some with noteworthy generalizations to language tests<sup>43,85,86</sup>. Additionally, multisensory training has been shown to increase temporal acuity in audiovisual integration<sup>87</sup>. Training paradigms aimed at increasing frequency resolution, multisensory integration, and sound localization are promising avenues for testing the benefits of perceptual training in CI users<sup>88</sup>.

## Conclusions

There is substantial evidence for multisensory integration in CI users that may have even greater perceptual benefit than what is experienced in normal hearing individuals. During periods of auditory deprivation and after cochlear implantation, unique sensory processing networks can form. As the criteria for CI candidacy broaden<sup>89</sup>, and the number of recipients approaches half a million individuals worldwide, there is a growing need to identify structural changes in sensory processing to better predict their compensatory or maladaptive influence on postoperative outcomes. Future work in this area will further elucidate underlying sensory deficits responsible for variability

in both audio-only and audiovisual integration.

Understanding how greater audiovisual gain leads to functional benefits to speech-in-noise intelligibility could directly inform preoperative consultations as well as currently implemented postoperative therapies. Specifically, there are therapies currently in practice that discouraging speechreading, instead emphasizing strict auditory-based rehabilitation (i.e., auditory-verbal therapy). These strategies may be underestimating the potential for audiovisual rehabilitation, while broadening this intervention to the visual domain might extend perceptual benefits.

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# A Review of Functional Domains Located Within the GABA<sub>A</sub> Subunit

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## Abstract

Ligand-gated  $\gamma$ -aminobutyric acid (GABA) receptors (GABA<sub>A</sub>Rs) are the primary mediators of fast synaptic inhibition in the brain. Of the 19 homologous genes that encode subunits for the GABA<sub>A</sub>R, three classes are typically assembled into pentameric heteromers, forming a receptor containing two  $\alpha$ , two  $\beta$ , and one  $\gamma$  or  $\delta$  subunit. All subunits have a similar topology with the following domains: a large, highly-structured N-terminal extracellular domain; four transmembrane domains, M1-M4; one extracellular loop, M2-M3; two intracellular loops, M1-M2 and the large, unstructured M3-M4; and a variable length extracellular C-terminal tail. Each domain serves a different, and sometimes more than one, functional role for the receptor as a whole. In addition, complex interactions may occur between multiple domains or multiple subunits, particularly surrounding agonist or allosteric modulator binding. The intricacies of each domain's functions will be explored here.

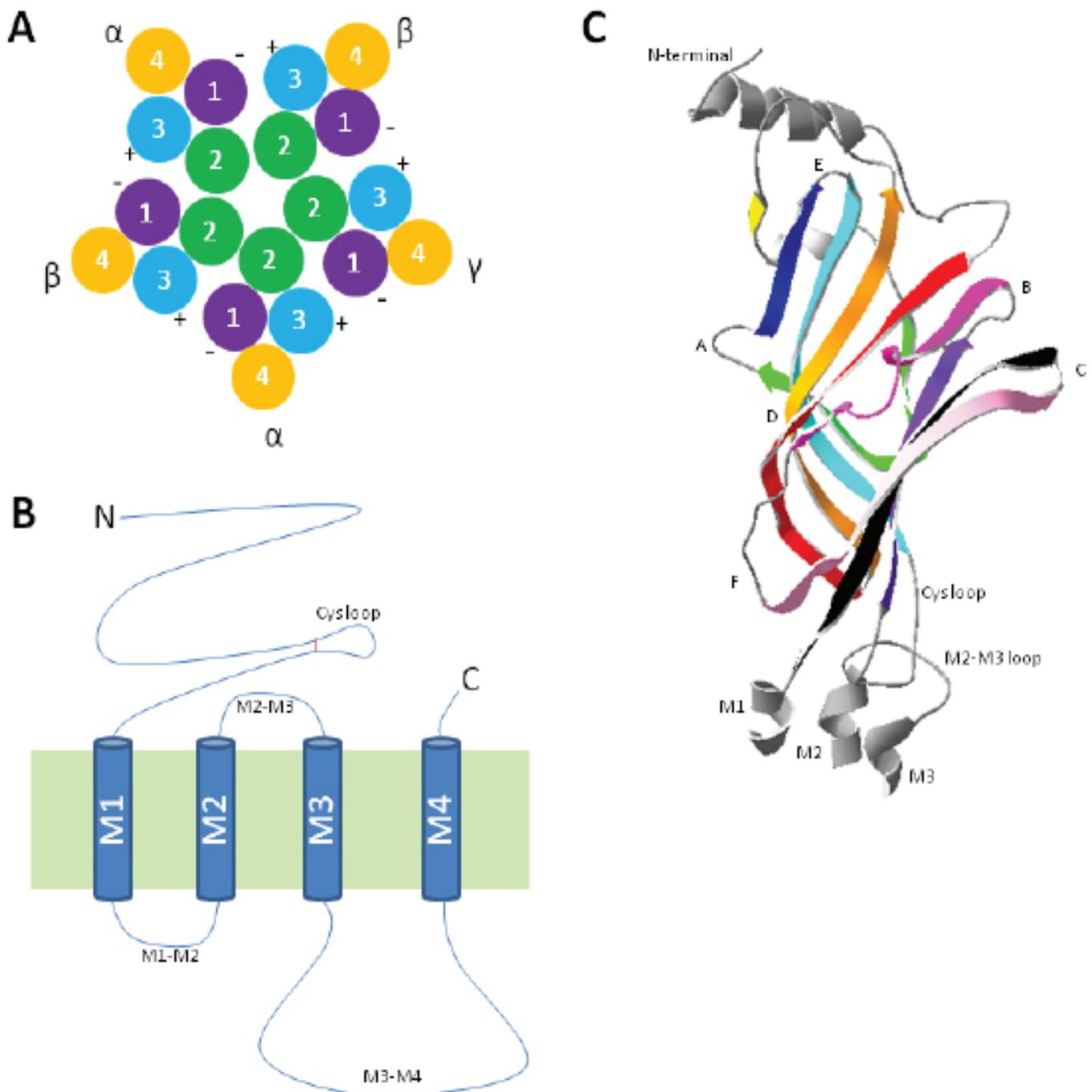
**Key words:** GABA, GABA<sub>A</sub>R, LGIC, glycosylation, anesthetic, benzodiazepine

## Introduction

GABA ( $\gamma$ -aminobutyric acid) is the primary signaling molecule for synaptic inhibition in the brain. GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) mediate fast inhibitory neurotransmission by conducting chloride ions into the cell. Epileptic encephalopathies can develop when GABA<sub>A</sub>Rs are functionally impaired. GABA<sub>A</sub>Rs are part of the ligand-gated ion channel superfamily, which consists of heteropentameric receptors that are permeable to ions when a ligand is bound<sup>1</sup>. There are 19 genes encoding subunits that can comprise GABA<sub>A</sub>Rs. These subunits all have a large N-terminal extracellular domain, followed by four transmembrane domains (M1-M4), with a large intracellular loop between the third and fourth transmembrane domain. While all GABA<sub>A</sub>R subunits are homologous, they have been divided into classes as follows:  $\alpha$  (1-6),  $\beta$  (1-3),  $\gamma$  (1-3),  $\delta$ ,  $\epsilon$  (1-3),  $\theta$ ,  $\pi$ , and  $\rho$ (1-3)<sup>2,3</sup>. GABA<sub>A</sub>Rs are typically ternary, comprised of two  $\alpha$ , two  $\beta$ , and one  $\gamma$  or  $\delta$  subunit<sup>4</sup>, arranged counterclockwise as  $\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\gamma$  when viewed from the extracellular space<sup>5</sup>. (**Fig. 1A**) GABA<sub>A</sub>Rs are a member of the cys-loop ligand gated ion channel (LGICs) family of receptors. All LGICs are all comprised of five homologous subunits that assemble around a membrane-spanning pore

through which ions may pass<sup>6</sup>. Members of the LGIC superfamily include the nicotinic acetylcholine receptor (nAChR), the prokaryotic homologs ELIC and GLIC7, the serotonin 5-HT<sub>3</sub> receptor, and the glycine receptor (GlyR)<sup>8</sup>. Recent work in crystallizing the GABA<sub>A</sub>R by Miller and colleagues has further elucidated many of the structural and functional complexities of this receptor<sup>3</sup>, whereas many previous studies had relied on the structural similarity between GABA<sub>A</sub>Rs and other LGICs.

Different subunits can confer different properties to the GABA<sub>A</sub>R, and the various regions within each subunit serve different functions to both the subunit and to the receptor overall. These region-specific functions will be discussed in detail here. The regions to be addressed are the large extracellular N-terminal domain, each of the four transmembrane domains (M1-M4), the loops between each of the transmembrane domains, and the variable length C-terminal tail. (**Fig. 1B**) First, general functions of each individual domain will be detailed. More complicated interactions, such as those involving more than one subunit, or those that occur at multiple locations within a single subunit, will then be discussed.



**Fig. 1:** A) Top-down view of the transmembrane domain arrangement of a fully assembled  $\alpha\beta\gamma$  GABA<sub>A</sub>R. The transmembrane domains are labeled by their number in order from the N-terminal to the C-terminal. M2 from each subunit faces the inside of the receptor, forming the pore-lining segments. The principle (+) and complementary (-) side of each subunit is indicated. B) 2D topographic representation of a single GABA<sub>A</sub>R subunit. Included are the extracellular N-terminal domain, the cys-loop, transmembrane domains M1-M4 and their corresponding linker loops, extracellular C-terminal tail. C) 3D model of a single GABA<sub>A</sub>R subunit. For clarity, only the extracellular domains and the most extracellular end of each transmembrane domains is shown. Additionally, M4 has been completely removed to allow for better visualization of the M2-M3 loop. B sheets are colored in the following order:  $\beta$ -1) red,  $\beta$ -2) orange,  $\beta$ -3) yellow,  $\beta$ -4) green,  $\beta$ -5) royal blue,  $\beta$ -6) cyan,  $\beta$ -7) violet,  $\beta$ -8) fuchsia,  $\beta$ -9) light pink,  $\beta$ -10) black. There are two extracellular  $\alpha$  helices ( $\alpha$ -1 and  $\alpha$ -2) towards the N-terminal of the extracellular domain. Loops important for forming the GABA binding pocket are as follows: on the principal (+) face of the  $\beta$  subunit: loop A, loop B, loop C; on the complementary (-) face of the  $\alpha$  subunit: loop D, loop E, loop F.

## N-Terminal Domain

The N-terminal of each subunit contains between 200 and 250 amino acids. The N-terminal domain of the GABA<sub>A</sub>R is highly structured (**Fig. 1C**), with an N-terminal  $\alpha$ -helix ( $\alpha$ -1), followed by ten  $\beta$ -sheets ( $\beta$ -1 –  $\beta$ -10), which fold and interact with one another. There is a second  $\alpha$ -helix ( $\alpha$ -2), between the  $\beta$ -3 and  $\beta$ -4, which aligns under  $\alpha$ -1. In a fully assembled receptor, the extracellular N-terminal domains form a ring around a pore, allowing water and solutes to access the transmembrane pore formed by the transmembrane domains discussed below. The N-terminal domain residues that line this pore are negatively charged, suggesting they may be modified by cations (e.g. zinc)<sup>3</sup>.

In adjacent  $\alpha$  and  $\beta$  subunits, two residues each in  $\alpha$ -1 are thought to interact by forming salt-bridges to encourage their thermodynamically favorable assembly. Mutations in the linker between  $\alpha$ -1 and  $\beta$ -1 can be associated with epilepsy, likely because they perturb the ability of subunits to properly assemble.  $\beta$ -4,  $\beta$ -5, and  $\beta$ -6 are also involved in allowing stable subunit assembly as they form interactions with neighboring subunits<sup>3</sup>. Mutations in the N-terminal domain that still allow for full subunit translation can have drastic effects on GABAergic signaling stemming from impaired receptor assembly and retention in the endoplasmic reticulum<sup>9</sup>.

### Glycosylation

The process of glycosylation occurs first in the endoplasmic reticulum where glycans are attached to the side chain nitrogen in asparagines. These glycans are thus known as N-linked. In order for an asparagine to be N-glycosylated, it must be located within the glycosylation consensus sequon: Asn-Xaa-(Ser/Thr), where Xaa is any amino acid except proline. Sequons containing a threonine in the third location are more likely to be glycosylated than those containing a serine. Glycosylation serves an important role for GABA<sub>A</sub>Rs, promoting proper folding of individual subunits, and promoting proper assembly of receptors from those subunits. In this way, glycosylation prevents aggregation and degradation of newly synthesized sub-

units. Additionally, glycosylation may affect subunit stoichiometry in assembled receptors, as well as function properties of those assembled receptors. Perturbations in glycosylation that either decrease (more typical) or increase the rate of glycosylation can lead to disrupted GABAergic signaling<sup>10</sup>.

The number of glycan groups differs per subunit, with the  $\beta$ 3 subunit containing three sites for N-linked glycosylation. The third glycosylation site on the  $\beta$ 3 subunit is hypothesized to strengthen the interaction between  $\beta$ -9/10 and  $\beta$ -7, and to facilitate the conformation change transduction of agonist binding to the channel pore<sup>3</sup>. However, the  $\alpha$ 1 subunit contains two potential glycosylation sites. Proper glycosylation of both of these sites is required for the subunit to properly assemble into a receptor<sup>11</sup>.

### Pre-M1 segment

The Pre-M1 segment is defined as the residues that make up the C-terminal of the final extracellular  $\beta$ -sheet ( $\beta$ -10) and the  $\beta$ -10 to M1 linker. This region serves several important roles for the GABA<sub>A</sub>R. It is thought to undergo structural changes when GABA is bound to the receptor, conferring part of the mechanism of activation of the receptor. Mutations in  $\beta$ -10 of the  $\alpha$ 1 subunit can shift the GABA response curve of the receptor to the right as compared to wild-type  $\alpha$ 1 $\beta$ 2 $\gamma$ 2S receptors, indicating a reduction in the affinity of the ligand for the mutant receptor. This led to a slower opening rate constant and a reduction of time spent in the open state for these mutant receptors<sup>12</sup>.

A cluster of positively charged amino acid residues comprise the pre-M1 segment, one of which is conserved across all GABA<sub>A</sub>R subunits, and across other LGICs. Mutations of these residues, converting lysine or arginine to cysteine, in either the  $\alpha$ 1 or  $\beta$ 2 subunit, can abolish channel gating, indicating a connection between GABA binding and gating that is fulfilled by this domain<sup>13</sup>.

## M1

Residues located in M1 of the  $\alpha$  subunit have been implicated in diverse interactions, including barbiturate binding and neurosteroid binding. Residues here are also believed to play a role in transducing

the binding of extracellular GABA to the pore-lining regions that gate the channel<sup>5</sup>, in conjunction with the role of the pre-M1 segment discussed above.

## M1-M2 Loop

The M1-M2 loop is a short, intracellular linker that connects the first two transmembrane domains. Mutational studies have revealed that several amino acid residues located in the M1-M2 linker of the  $\beta$  subunit influence the ion selectivity of the GABA-

$\text{A}_\text{A}$ R. In fact, mutations in the M1-M2 linker of the  $\beta$  subunit are sufficient to render the GABA $\text{A}_\text{A}$ R selective for cations, as opposed to the typical chloride anion selectivity of the wild type receptor<sup>14</sup>.

## M2

M2, which forms the channel-lining segment, is highly conserved across all GABA $\text{A}_\text{A}$ R subunits. The subunits that line the pore were first identified by serial substitution of individual amino acids in M2 with cysteines<sup>15</sup>. Some subunits contain minor sequence differences, which are thought to confer zinc sensitivity in the  $\gamma$  subunit. Several of these amino acid residues are water accessible. The M2 domain of the  $\beta$  subunit is so important for ion gating that substituting M2 of the  $\beta$ 3 subunit with the M2 of either  $\alpha$ 2 or  $\gamma$ 2 yields a non-functional receptor<sup>6</sup>. However,  $\beta$  subunits are known to form functional homomers<sup>3</sup>, therefore the  $\beta$  M2 is necessary and sufficient for channel function.

The 3D structure of M2 has been demonstrated to be an  $\alpha$  helix that is widest at the extracellular

end and narrows as it approaches the cytoplasmic side, with a kink in the center of the channel<sup>14</sup>. This indicates that the “closed gate” of the receptor is towards the inside of the channel, as opposed to other LGIC structures which have their closed gates towards the extracellular side of the channel. Approximately halfway down M2, each subunit has a positively charged residue, which form a positively-charged ring. This ring presumably allows for the anion-selectivity of the GABA $\text{A}_\text{A}$ R. It is believed that desensitization is the result of a conformational change in residues lining the pore that result in their side chains being shifted towards the opening of the pore. Drugs that block desensitization may therefore act by limiting this conformational change, leaving the pore accessible<sup>3</sup>.

## M2-M3 Loop

The M2-M3 loop forms interactions with the N-terminal extracellular domain that are important for channel gating. The outer portion of the M2-M3 loop forms polar contacts with the loop connecting  $\beta$ -6 and  $\beta$ -7, where the cys-loop is located. Additionally, the inner portion of the M2-M3 loop (towards the pore), forms van der Waals contacts between the loops connecting  $\beta$ -1 and  $\beta$ -2, and  $\beta$ -6 and  $\beta$ -7. Salt bridges are also formed

between the M2-M3 loop and the cys-loop, which was mentioned previously to in turn have connections with the “closed lid” over the GABA binding pocket formed by loop C in the extracellular domain<sup>3</sup>. Thus, it is possible to see how a conformational change that begins in loop C is transmitted to the cys loop, then to the M2-M3 loop, and down into the channel lining segments (M2).

## M3

M3 forms several hydrogen bonds with the loop between  $\beta$ -6 and  $\beta$ -7 in the N-terminal extracellular domain, providing further structural coupling between the transmembrane domains and the N-terminal domain. Mutations affecting this coupling,

as well as the coupling to the M2-M3 loop, are often associated with epileptic encephalopathies, indicating their importance to normal receptor function<sup>3</sup>. M3 can be important for the actions of certain drugs, including anesthetics, to be discussed below.

### M3-M4 Loop

The M3-M4 loop varies in size between subunits, with the smallest loop containing only 85 amino acids, and the largest loop containing 255 amino acids. There is little information on the predicted structure of the M3-M4 loop, and in fact the loop had to be removed for successful GABA<sub>A</sub>R crystallization<sup>3</sup>. The evidence that does exist for the function of the M3-M4 loop is not without conflict. One of the primary roles of this domain is to interact with intracellular scaffolding proteins, such as gephyrin and collybistin. Gephyrin is involved in clustering GABA<sub>A</sub>Rs postsynaptically to enable the formation of inhibitory synapses<sup>17</sup>. Collybistin plays a more supporting role in that it induces gephyrin to cluster at the membrane<sup>18</sup>. Saiepour and colleagues demonstrated via co-immunoprecipitation that the M3-M4 loop of the  $\alpha$ 2 subunit is capable of forming a trimeric complex with both gephyrin and collybistin, which allowed

for submembrane aggregates to form, indicating this interaction could be important for GABAergic synapse formation<sup>19</sup>. However, other groups have shown that  $\alpha$ 320,  $\gamma$ 221,  $\gamma$ 322,  $\beta$ 2 and  $\beta$ 323 may play important roles as well. Other anchoring proteins, such as radixin<sup>24</sup>, may also interact with this domain. Clearly, more work needs to be done to clarify this complex interaction between GABA<sub>A</sub>R subunits and intracellular structural, anchoring, chaperone and regulatory proteins.

Phosphorylation events can also occur within the M3-M4 loop, which regulate the ability of GABA<sub>A</sub>R subunits to interact with intracellular protein complexes. These complexes can affect endocytosis and insertion of GABA<sub>A</sub>Rs at the cell membrane.  $\alpha$ 4, for example, appears to be phosphorylated by protein kinase C (PKC), which results in cell-surface accumulation of receptors containing this subunit<sup>25</sup>.

## M4

The role of the M4 domain is not fully understood. However, many anesthetics target the  $\beta$  subunit to execute their action, and propofol in particular has been shown to act on M4 in studies that mutated residues in M4<sup>26</sup>. Mutations in M4 of nAChR, a member of the LGIC family that is homologous to the GABA<sub>A</sub>R, cause subunits to be retained in the

ER instead of being trafficked to the cell surface. These mutations also disrupt assembly of mutant subunits with other, wild type subunits<sup>27</sup>. These data could indicate a role of the GABA<sub>A</sub>R M4 in receptor trafficking. Some data also indicate that M4 of the  $\gamma$ 2 subunit may be responsible for post-synaptic clustering of GABA<sub>A</sub>Rs and gephyrin<sup>28</sup>.

### C-Terminal Tail

Few studies have described the function of the C-terminal tail.  $\alpha$  subunits have the most significant C-terminal tail. Insertion of GFP into the C-terminal tail of the  $\gamma$ 2 subunit leads to sub-

stantial degradation and a barely detectable level of subunit expression<sup>29</sup>. It is possible this occurred because GFP affected subunit folding due to the rather short C-terminal tail on  $\gamma$ 2.

## Complex Interactions with Agonists, Drugs, and Metals

Many drugs, including anesthetics and anti-anxiolytics, interact with GABA<sub>A</sub>Rs at more than one discrete location. Often these interactions occur at the interfaces between two subunits, where

M3 of one subunit interacts with M1 of the adjacent subunit, what is called a +/- interface. (Fig. 1A) However, other agents, such as zinc, affect several regions of both the  $\alpha$  and the  $\beta$  subunit.

### *Zinc sensitivity*

Zinc is abundant in the brain and can be stored in synaptic vesicles, where it is released into synapses to serve a modulatory purpose after synaptic events. While zinc is not believed to be co-released with GABA, zinc is stored in glutamatergic synaptic vesicles and spillover from these glutamatergic synapses can influence inhibitory synapses. Zinc has been shown to reduce the amplitude, slow the rise time, and shorten the decay time of GABAergic currents with direct application<sup>30</sup>.

It has been demonstrated that the  $\gamma$  subunit reduces sensitivity of the GABA<sub>A</sub>R to zinc inhibition. Even with a 10 second pre-incubation with zinc,  $\alpha\beta\gamma$  receptors show little effect of zinc, whereas the current generated from binary  $\alpha\beta$  are greatly impacted<sup>31</sup>, as discussed above.  $\alpha\beta\delta$  receptors remain fairly sensitive to zinc inhibition. Zinc interaction domains have been identified in the  $\alpha$  and  $\beta$  subunits: in  $\beta$  homomers and in  $\alpha\beta$  binary receptors, the extracellular portion of M2 has been shown to interact with zinc. Within M2, there are just four different amino acids between  $\gamma$ 2L (zinc insensitive) and  $\delta$  (zinc-sensitive) towards the extracellular end of M2, which are hypothesized to cause this change in zinc sensitivity. In some trimeric receptors, the M2-M3 loop has been implicated in zinc sensitivity, primarily receptors containing the  $\alpha 6$  subunit. Using chimeric subunits, in which regions of one subunit are replaced with that of another subunit in an attempt to identify particular functional regions within a target subunit, the N-terminal and the outer mouth of the channel (M1-M2 loop and extracellular end of M2) have been identified in the  $\gamma$  subunit as being responsible for conferring a low zinc sensitivity to trimeric  $\alpha\beta\gamma$  receptors<sup>16</sup>.

### *Anesthetics*

GABA<sub>A</sub>Rs have been implicated as the effector for general anesthesia<sup>26</sup>. Etomidate, one of several intravenous general anesthetics, increases the affini-

ty of GABA for the GABA<sub>A</sub>R, enhancing receptor activation. Studies with photoreactive etomidate analogs identified residues in M1 of  $\alpha$  and M3 of  $\beta$  as potential binding sites for etomidate<sup>5,32</sup>. These residues are found along the  $\alpha$ -/ $\beta$ + interface. Additional studies have implicated M2 as forming part of the etomidate binding pocket<sup>33</sup>. Another intravenous anesthetic, propofol, has been shown to bind to interact with M3 of the  $\beta$  subunit<sup>34</sup>. Finally, barbiturates, yet another class of anesthetics, interact with the M1 region of the  $\beta$  subunit<sup>35</sup>, the (-) interface of the subunit, and with the adjacent M3 or (+) interface on  $\alpha$  and  $\gamma$  subunits<sup>7</sup>.

### *Benzodiazepine*

A high affinity positive allosteric modulator for GABA<sub>A</sub>Rs<sup>36</sup>, benzodiazepine is a drug that is used for its sedative, anxiolytic, anticonvulsant, and muscle relaxant properties<sup>37</sup>. Benzodiazepine induces these effects by binding to the GABA<sub>A</sub>R, inducing a conformational change in the receptor that increases the single-channel opening frequency of the receptor. The presence of a  $\gamma$  subunit is required in order for benzodiazepine to bind GABA<sub>A</sub>Rs. This binding occurs at the interface between the  $\gamma$  and the  $\alpha$  subunit in a trimeric  $\alpha\beta\gamma$  receptor, at a site homologous to the GABA binding site in the  $\alpha$ - $\beta$  interface discussed above. Replacement of the  $\gamma$  with a  $\delta$  subunit abolishes the ability of benzodiazepine to modulate GABA<sub>A</sub>Rs. Using a method similar to that used to elucidate the mechanism of action of zinc, chimeras were generated to contain specific and variable portions of either a  $\gamma$  or a  $\delta$  subunit in order to identify the functional domains responsible for benzodiazepine's action. The chimeric subunits were co-expressed with  $\alpha$  and  $\beta$  subunits to allow assembly into full receptors. The sensitivity of the receptors to benzodiazepines was assessed via whole-cell voltage-clamp recordings. A complex interaction was elucidated with this method: benzodiazepine interacts with the more extracellular portions of both M1 and M2, and the extracellular M2-M3 loop of the  $\gamma$  subunit<sup>2</sup>.

## Conclusion

GABA<sub>A</sub>Rs are quite complex, with a multitude of functional domains and possible complex interactions. The extracellular N-terminal domain is high structured and is critical for the binding of

GABA and for transducing the conformational change induced by agonist binding down to the transmembrane domains. The transmembrane domains each serve different roles, from lining the chloride ion-conducting pore, to diverse interactions with drugs and zinc. The inter-transmembrane domain loops serve variable roles. The M2-M3 loop serves the main role of transducing the agonist binding-induced conformational changes

to the pore of the receptor. The M3-M4 loop is not incredibly well characterized, especially given its length. However, it is known to interact with intracellular signaling molecules, such as PKC, and scaffolding or trafficking proteins, such as gephyrin. With such diverse and important roles, it is evident how mutations in any of these subunits may have substantial effects on GABAergic signaling, potentially leading to epileptic disorders.

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# The Role of Distinct Central Amygdala Neuronal Populations in the Regulation of Fear and Anxiety

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## Abstract

The amygdala plays a crucial role in the regulation of emotional learning and motivation. Specifically, the central nucleus of the amygdala has been hypothesized to serve as a gate for the generation of conditioned fear and related anxiety behaviors. The central amygdala can be anatomically divided into subregions, which contain functionally distinct neuronal populations capable of bidirectional control over the expression of defensive behaviors. The recent use of transgenic reporter mouse lines, combined with optogenetic and chemogenetic techniques, have allowed researchers to begin to characterize how neurochemically distinct neuronal populations fit within the functional microcircuitry of the central amygdala. Herein, I summarize recent findings demonstrating how neurochemically identified neuronal populations in the central amygdala fit within the functional and anatomical framework to either promote or inhibit the expression of fear and anxiety behaviors

**Key words:** *amygdala, fear, anxiety, optogenetic, chemogenetic, transgenic.*

## Introduction

The behavioral manifestations of fear and anxiety are profoundly conserved across mammalian species. Observations from model organisms have demonstrated reliable defensive behaviors, such as freezing, avoidance, and escape from stressful or negatively arousing environmental stimuli<sup>1,2</sup>. Thus, research that aims to measure defensive behaviors has served as a way of assessing the level of fear or anxiety in animals. For example, a classically used paradigm in rodent research is Pavlovian ‘fear conditioning.’ Fear conditioning consists of a learned association between a generally aversive unconditioned stimulus (US) and an otherwise neutral conditioned stimulus (CS)<sup>3</sup>. Typically, <sup>5-6</sup>temporal pairings of an US, such as a foot-shock, and a CS, such as an auditory tone, can reliably produce a long-lasting defensive freezing response in rodents upon re-exposure to the CS alone<sup>4</sup>. Therefore, freezing behavior to the CS represents a state of learned fear in a rodent, which can be broken down into its neural and molecular correlates and explored in depth using a wide variety of neuroscience research techniques. In addition, exposure to a stressful stimulus, such as a foot-shock, can cause an increase in anxiety. Anxiety can then be measured by examining a rodent’s exploration of novel

environments and avoidance of naturally aversive stimuli. Although generally defined as different behavioral states, the physiological symptoms and behavioral expression of fear and anxiety may be mediated by overlapping neural circuitry<sup>5</sup>, some of which will be discussed in this review. Because the expression of fear and anxiety is beneficial for the survival of many organisms, the underlying neural circuitry and molecular substrates that mediate these behaviors are likely to have been sculpted by evolution and retained across species.

One critical brain region that has been implicated in the regulation of fear and anxiety is the amygdala. The amygdala plays a crucial role in the regulation of emotional learning, anxiety induction, and motivational behaviors, making it an important structure at the interface between stress exposure and behavioral output. The amygdala can be anatomically divided into a number of nuclei that are designed to receive, encode, or relay information about stressful or potentially threatening experiences. Briefly, the lateral amygdala (LA) serves as a main input structure, receiving excitatory afferents from the thalamus and multiple association cortical areas, and plays a large role in integrat-

ing CS sensory information with US information during fear conditioning<sup>6-8</sup>. Similarly, the basolateral amygdala (BLA) receives substantial input from a number of brain structures of cortical origin, including the LA and hippocampus, as well as top down cognitive control of learned fear and anxiety via excitatory inputs from the medial prefrontal cortex (mPFC)<sup>9</sup>. The central nucleus of the amygdala (CeA) receives visceral, nociceptive, and polymodal sensory information from cortical and subcortical brain regions. However, the CeA differs from the LA and BLA in that it is the primary output region of the amygdala, sending efferent projections to downstream brainstem nuclei that are responsible for the induction of autonomic and defensive behavioral responses. These nuclei of the amygdala are necessarily interconnected to allow for the tight control of fear and anxiety acquisition, retention, and expression. This interconnectivity occurs in a dorsoventral and lateromedial architecture, where the LA primarily projects to the BLA and CeA, and the BLA primarily projects to the CeA<sup>10</sup>.

Although the differences in functional organization between amygdalar nuclei have been dis-

cussed in great detail in other reviews, this review will focus on the CeA, which can be described as a 'gate' for the expression of fear and anxiety. Specifically, recent advances in neuroscience techniques over the past decade have allowed researchers to begin to further characterize the role of individual cell-types in the regulation of fear and anxiety, allowing for a greater level of analysis into how the CeA gates conditioned fear and anxiety behaviors. Therefore, this review will highlight recent findings into the role of individual neuronal populations in the CeA, starting with an overview of anatomically defined CeA neurons, functionally defined CeA neurons, and finishing with the neurochemically defined CeA neurons that may fit into part of the finely tuned functional microcircuitry of the CeA. Due to the limited amount of research regarding which CeA cell-types regulate fear and anxiety, specific attention to recent publications that have neurochemically addressed this question will be emphasized. Finally, future directions of research into cell-type specific CeA microcircuitry will be briefly discussed in relevance to this rapidly emerging line of investigation.

## Anatomically Defined Cell Populations of the CeA

The CeA can be further divided into anatomical subregions, consisting of the capsular division (CeC), the lateral division (CeL), and the medial division (CeM). In most cases, researchers consider the CeC to be part of the CeL, with very little functional distinction between the two, and as such these two subregions will be collectively defined as the CeL throughout this review. The CeL and CeM exclusively consist of GABAergic neurons of striatal origin<sup>11</sup>. CeL neurons are anatomically similar to medium spiny neurons of the striatum, and are characterized by their multiple branching dendritic processes, high expression of spines, and small somata<sup>12</sup>. However, CeM neurons express larger somata, minimally branching dendrites, and much smaller density of dendritic spines than the CeL. Despite the neuroanatomical differences of the CeL and CeM neurons, neurons from both subregions have heterogeneous physiological firing properties, which have been separated into three classes:

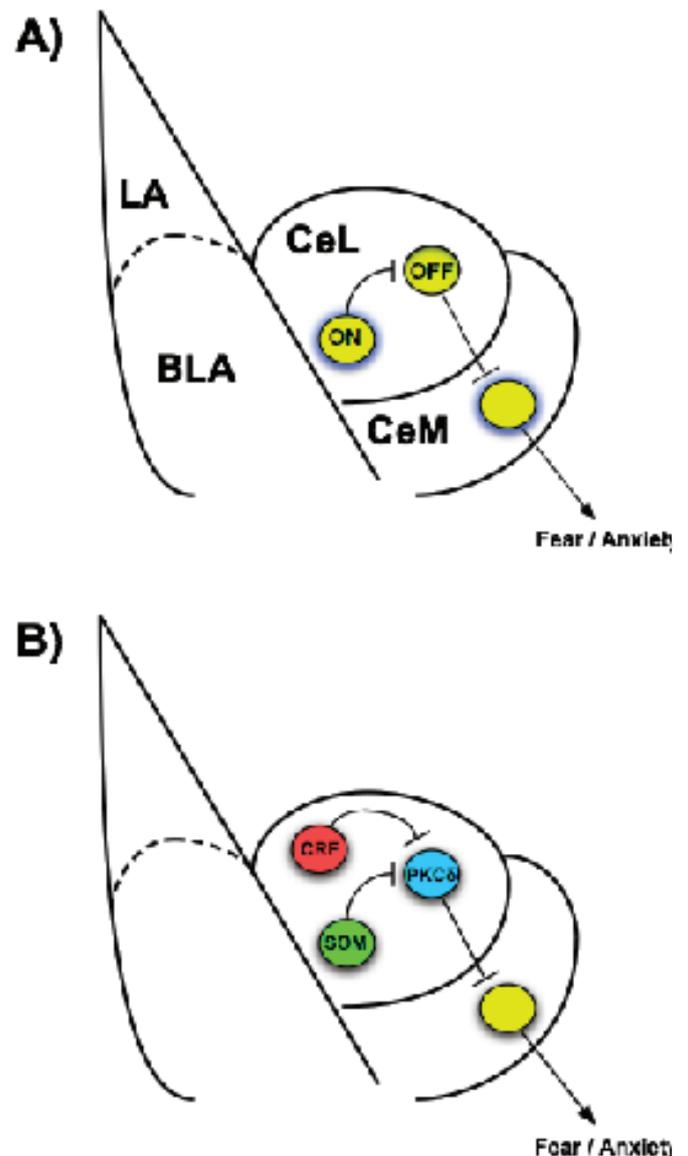
late firing, regular firing, and low threshold bursting<sup>13</sup>. Although a small population of CeL neurons expresses long-range projections, the majority projects either locally within the CeL or directly to the CeM, whereas the primary output of the CeA occurs via the principal neurons of the CeM. The principal CeM neurons have been shown to project to downstream effector nuclei such as the bed nucleus of the stria terminalis (BNST), the lateral hypothalamus (LH), the dorsal vagal complex (DVC), and the periaqueductal gray (PAG), all of which contribute to autonomic and behavioral responses associated with anxiety and fear<sup>14</sup>. Perhaps the most relevant to immediate or highly salient threats is the PAG. The PAG is necessary for the expression of freezing behavior in fear conditioning, and is linked to a number of defensive anxiety phenotypes<sup>15</sup>. Therefore, much of the literature has focused on populations of neurons that project to this region, and the role they play in conditioned fear.

## Functionally Defined Cell Populations of the CeA

As described above, the CeA is believed to gate behavioral expression of fear and anxiety. This process occurs through functionally distinct inhibitory circuits involving the CeL and CeM. Generally, the CeM mediates the expression of fear and anxiety behaviors, while the CeL inhibits CeM output (Fig 1A). Previous reports using retrograde and anterograde tracer studies and immunohistochemical analyses have supported an anatomical architecture where CeL neurons send projections to the CeM but the CeM does not send significant projections back to the CeL<sup>16-18</sup>. Consistent with this hypothesis, Ciocchi and colleagues in 2010 demonstrated how CeM neurons in fear conditioned mice showed sustained increases in firing rate during in vivo electrophysiological recordings following presentation of an auditory CS. Directed optogenetic activation of the CeM using channel rhodopsin (ChR2) also elicited freezing behavior, whereas pharmacological blockade of the CeM using the GABA<sub>A</sub> receptor agonist muscimol prevented the expression of conditioned freezing responses<sup>19</sup>. In accordance with the inhibition of CeM output by the CeL, Ciocchi et al. also demonstrated how inhibition of the CeL using muscimol caused unconditioned freezing responses in naive mice. Therefore, these findings support the idea that CeL neurons prevent the expression of freezing behavior via tonic inhibition onto CeM neurons, while direct activation of CeM neurons following fear conditioning is responsible for eliciting freezing responses during exposure to the CS. Regardless of these interesting findings, the empirically derived circuit between the CeL and CeM raises an interesting question: how does the activity of CeL neurons gate CeM output?

Surely, if the CeL were involved in tonic inhibition of the CeM, then it would be expected that CeL neurons must be inhibited during re-exposure to the CS in order to allow activation of CeM neurons. However, this concept is somewhat oversimplified in regard to the regulation of CeL neuronal activity in fear learning. For example, in vivo recordings of CeL neurons indicate separate populations, with differing responses to the CS following fear conditioning. As compared to

pre-training baseline recordings, one population of neurons in the CeL shows increased activity during CS presentation (CeL-On cells), whereas another population demonstrates decreased activity following CS presentation (CeL-Off cells)<sup>19</sup>. This unique finding suggests a potential model where CeL-On cells may be responsible for local inhibition of CeL-Off cells, and that CeL-Off cells may specifically target the CeM over CeL-On



**Fig. 1.** Functional CeA microcircuitry regulating fear and anxiety. A) CeL-On cells promote fear and anxiety expression through disinhibition of the CeM, and CeL-Off cells prevent fear and anxiety expression through tonic inhibition of the CeM. B) Hypothetical model of neurochemically defined CeL-On and CeL-Off cell populations.

cells (**Fig. 1A**). In concert with this hypothesis, an analysis of stimulus onset firing latency has demonstrated that CeL-On cells are activated before the decrease in activity of CeL-Off cells, and that the number of CeL-Off cells near-

ly triples over 24 hours whereas the number CeL-On cells remains roughly constant<sup>19,20</sup>. Overall, these data suggest an overarching microcircuitry within the CeA that could regulate the expression of defensive behaviors (**Fig. 1A**).

## Neurochemically Defined Cell Populations of the CeA

Neurons within the CeA contain a wide range of co-localizing and non co-localizing co-transmitters, neuropeptides, and protein markers. The recent use of transgenic reporter mice, optogenetic techniques, and chemogenetic techniques have allowed researchers to explore subpopulations of neurochemically defined CeA neurons, and how these neurons may fit within the previously described functional circuitry of the CeA. A few of these neurochemically-defined populations and their relevance to fear and anxiety will be described below. Specific detail will be provided on recent studies that have thoroughly characterized populations of cells, but a review of other populations will also be discussed.

### *Protein Kinase C- $\delta$*

Protein Kinase C- $\delta$  (PKC $\delta$ ) is a phospholipid dependent protein kinase C isoform that may play a large role in neuronal plasticity and regulation of intracellular signaling cascades<sup>21</sup>. PKC $\delta$  expressing neurons in the CeA (PKC $\delta$ +) are restricted to the CeL, and comprise about 50% of CeL neurons<sup>22</sup>. The anatomical, physiological, and functional properties of this population of neurons was first characterized by Haubensak and colleagues in 2010 using a bacterial artificial chromosome (BAC) transgenic reporter line that expresses Cre-recombinase (Cre)<sup>b</sup> and a cyan fluorescent protein (CFP) tagged invertebrate-sensitive glutamate-gated chloride channel (GluCl $\alpha$ ; mutated to remove glutamate sensitivity) under the expression of the PKC $\delta$  promoter. This method allowed the authors to identify fluorescent PKC $\delta$  neurons throughout the brain via their expression of CFP. In addition, the method allowed the authors to use Cre-lox technology to selectively manipulate Cre-expressing PKC $\delta$  neurons via adeno-associated virus (AAV)<sup>c</sup> delivery of a GluCl $\delta$  subunit into the CeL, conferring functional heterodimeric for-

mation of the invertebrate sensitive GluCl $\alpha\beta$  channels only in PKC $\delta$  neurons. PKC $\delta$  neurons in the CeL were found to consist of each of the physiologically defined classes found throughout the CeA: late firing, regular firing, and low-threshold bursting neurons, with the vast majority consisting of late firing neurons. Interestingly, by analyzing the quantity of CeL-On and CeL-Off neurons using in vivo unit recordings, the authors discovered that inhibition of PKC $\delta$  neurons with invertebrate-sensitive channels decreased the tonic spontaneous activity of CeL-Off neurons, whereas CeL-On neurons were unaffected. Moreover, inhibition of the PKC $\delta$  neurons using this method resulted in increased firing rates from CeM unit recordings. These findings imply that PKC $\delta$  neurons could potentially represent the functionally defined CeL-Off cells identified in previous studies, which can suppress CeM output (**Fig. 1B**). In support of this hypothesis, the use of retrograde and anterograde tracers identified that PKC $\delta$  cells primarily project to the CeM, and that inhibition of PKC $\delta$  neurons increased freezing responses to a CS following conditioned-fear training<sup>22</sup>. Although the behavioral effect of silencing PKC $\delta$  neurons implicates their inclusion as members of the functional class of CeL-Off cells, unconditioned freezing behavior was not increased when these neurons were inhibited prior to training. This result differs from the Cioocchi et al. findings, where pharmacological inhibition of the CeL with muscimol caused increases in unconditioned freezing behavior of naive mice (although these differences may arise due to the application of different techniques). Despite this difference, the findings still provide strong evidence that PKC $\delta$  neurons are CeL-Off neurons. However, it is important to note that this finding does not directly suggest that all CeL-Off cells are neurochemically defined as PKC $\delta$ +, and further research is needed to identify whether other cell-types in the CeL may represent CeL-Off cells as well.

Providing support for the role of PKC $\delta$ + neurons in CeA microcircuitry, Cai and colleagues in 2014 found that PKC $\delta$ + neurons form monosynaptic inhibitory synapses onto PKC $\delta$ - neurons, suggesting that there are functional connections between CeL-On and CeL-Off cells. In agreement with the previous study, activation of PKC $\delta$ + neurons with Chr2 caused an increase in percent open arm time in the elevated plus maze, and an increase in time spent in a light chamber in the light-dark box test, two measures that reliably indicate a decrease in anxiety in rodents. Thus, the relative activity of PKC $\delta$ + neurons may determine the level of emotional arousal in animals, where PKC $\delta$ + activity could prevent fear expression by inhibiting PAG projecting CeM neurons, or by decreasing the overall level of anxiety. Whether generalized anxiety and conditioned fear are entirely controlled by the same circuitry has not yet been conclusively determined; still, these findings suggest that the CeA microcircuitry is positioned to regulate the expression of both conditioned fear and anxiety, and that cell-type specific activity levels in the CeA may bi-directionally control related behaviors.

Although PKC $\delta$ + neuronal activity can exert control over fear and anxiety expression, the role of this neuronal population may be even more complex than previously thought. Cai et al. also found that the activity of PKC $\delta$ + neurons mediates anorexigenic signals and that PKC $\delta$ + neurons demonstrate marked increases in c-Fos expression following administration of compounds that evoke satiety, sickness, nausea, or visceral malaise<sup>23-26</sup>. Indeed, optogenetically activating these neurons with Chr2 caused a substantial decrease in feeding behavior in mice, whereas inhibiting these neurons using halorhodopsin (eNpHR3.0), the light-activated chloride pump, or a G $\alpha$ i-coupled designer receptor exclusively activated by designer drugs (G $\alpha$ i-DREADD)d, caused an increase in feeding behavior in satiated animals. These findings are particularly surprising given the relationship between anxiety and feeding behavior, as well as the overlap in representative brain structures that control these processes<sup>27</sup>. Typically, stressed or anxious animals demonstrate marked decreases in feeding behavior, so it is intriguing that PKC $\delta$ + neurons ap-

pear poised to decrease fear expression while still capable of increasing anorexic behavior. If PKC $\delta$ + neurons are functionally defined as CeL-Off cells, which prevent fear and anxiety expression, then data from this study raises the question of why activation of these neurons would cause substantial decreases in food consumption. Perhaps these neurons can be further divided into non-overlapping populations of CeL-Off neurons that tonically inhibit fear or neurons that inhibit feeding, which could receive different afferent inputs and send efferent projections to different brain structures. In support of this notion, 40% of PKC $\delta$ + neurons also co-express the neuropeptide enkaphalin, which is an opioid peptide associated with the regulation of nociception<sup>28</sup>. Therefore, there may be subpopulations of neurochemically defined PKC $\delta$ + neurons that serve diverse roles, but future experiments into the synaptic inputs and outputs of these neurons will be needed to provide insights into the purpose they serve within CeA microcircuitry.

#### *Somatostatin*

In the central nervous system (CNS), somatostatin (SOM) serves as an inhibitory neuropeptide hormone that plays a large role in neuronal communication throughout the brain via its actions on G-protein coupled SOM receptors<sup>29</sup>. Application of SOM onto ex vivo rat brain slices of the CeA can depress activity of CeA neurons by increasing potassium conductance<sup>30</sup>. Thus, neuronal release of SOM within the CeA could serve as an additional form of inhibition that is secondary to the fast GABAergic neurotransmission that dominates CeA microcircuitry. Li and colleagues in 2012, were the first to specifically characterize SOM expressing neurons (SOM+) in the CeA. In order to conduct functional and physiological analyses of SOM+ neurons, the authors used a transgenic mouse line that drives the expression of Cre under the SOM promoter (SOM-IRES-Cre). They crossed this mouse line to a reporter line (Ai14)e, which allows the expression of the fluorescent protein TdTomato in a Cre-dependent manner, only in SOM+ neurons. The SOM+ neurons were heavily restricted to the CeL, and following immunohistochemical analysis, were found to be majorly non-overlap-

ping with PKC $\delta$ + neurons, indicating that SOM+ neurons are not likely part of the CeL-Off cell population. Furthermore, physiological recordings of these neurons indicated that they fell into the class of late firing and regular firing neurons.

Li et al. also demonstrated how chemogenetic inhibition of SOM+ neurons in the CeL, using AAV delivery of a Cre-dependent Gq $\alpha$ -DREADD, prevented the acquisition of conditioned fear. Alternatively, activation of SOM+ neurons using Cre-dependent ChR2 caused unconditioned freezing in naive mice. Taken together, these findings propose that SOM+ neurons may fall into the class of CeL-On cells (**Fig. 1B**). If these neurons consist of CeL-On cells then one might expect their firing rates to increase following presentation of a CS in fear-conditioned mice. However, the easy identification of specific neurochemically defined cell-types using in vivo unit recordings during real time is not yet technologically feasible. To circumvent this issue, the authors used ex vivo electrophysiological analyses of synaptic plasticity onto SOM+ neurons. Up until this point in time, fear-conditioning induced synaptic plasticity in the CeL had not been demonstrated, and was believed to be restricted to the LA and BLA. For the first time, the authors found that fear conditioning induced significant increases in excitatory postsynaptic current (EPSC) frequency, as well as AMPA and NMDA receptor mediated EPSC amplitudes, onto SOM+ but not SOM- neurons during paired recordings. The presynaptic component of this plasticity could be recapitulated by stimulation of LA neurons using ChR2, indicating that SOM+ neurons are indeed implicated in the canonical fear circuitry of the greater amygdala. Tracer injections and an analysis of inhibitory postsynaptic currents (IPSCs) from CeM neurons further indicated that SOM+ neurons do not project to the CeM, consistent with their classification as CeL-On cells<sup>31</sup>. Cumulatively, this study thoroughly demonstrates that SOM+ neurons in the CeA are intimately involved in the acquisition and expression of conditioned fear, and are candidate neurons for the class of CeL-On cells (**Fig. 1B**).

Although SOM+ neurons express fear conditioned synaptic plasticity from glutamatergic LA inputs,

a recent study by Penzo and colleagues in 2015 demonstrated that SOM+ neuronal plasticity also occurs via projections from the paraventricular nucleus of the thalamus (PVT). These PVT to CeL inputs preferentially synapse onto SOM+ neurons as opposed to SOM- neurons and mediate fear expression via BDNF signaling<sup>32</sup>. In fact, ChR2 mediated excitation of PVT terminals in the CeL did not evoke fast neurotransmission but rather slow inward currents following high-frequency stimulation, consistent with the effects of a neuromodulator such as BDNF. These findings advance the current understanding of CeA cell-type modulation of fear and anxiety because they suggest that the CeA may not just serve as a 'gate' for fear expression, but may also be actively involved in the storage and retention of fear memories. Curiously, a small population of SOM+ neurons in the CeL also send long range projections to the PAG and PVT, or both, further complicating the functional scheme of SOM+ neurons<sup>33</sup>. Therefore, future research is still needed to address the multitude of functional inputs and outputs of neurochemically defined CeA populations in order to shed light on potential discrepancies to the current hypothetical model (**Fig. 1B**), or elucidate further intricacies within the framework.

#### *Corticotropin Releasing Factor*

Corticotropin releasing factor (CRF) is an excitatory neuropeptide hormone that is involved in the regulation of feeding behavior, audition, cardiac function, and the stress response<sup>34</sup>. Importantly, CRF has been significantly implicated in regulating physiological stress responses due to its action upon CRF receptors in the paraventricular nucleus of the hypothalamus, the BNST (part of the extended amygdala), and the CeA. As an extension of its direct role on neuroendocrine cells of the paraventricular nucleus of the hypothalamus, CRF levels are elevated in the brains of psychiatric patients suffering from post-traumatic stress disorder (PTSD) and related anxiety disorders<sup>35,36</sup>. This finding suggests that CRF expressing neurons (CRF+) may play an important role in pathological states of elevated fear and anxiety. In agreement with this finding, over-expression, microinfusion, or receptor agonism in the CeA

sion, microinfusion, or receptor agonism in the CeA causes an increase in anxiety and defensive behaviors in rodents<sup>34,37-39</sup>. Conversely, genetic disruption of CRF signaling via CRF knockdown or CRF receptor antagonism has anxiolytic effects, and attenuates the augmentation of anxiety that occurs following exposure to traumatic environmental stressors<sup>40-43</sup>. Likewise, administration of CRF antisense oligonucleotides can prevent the consolidation of conditioned fear<sup>44,45</sup>.

In situ hybridization and the recent use of mouse transgenic reporter lines have aided in the identification of CRF+ neurons in the BNST and CeA, making local release of CRF from these neurons a likely candidate for amygdalar control over fear and anxiety expression. A commercially available CRF-IRES-Cre line has been used by researchers, which can be crossed to an Ai9 reporter line, in order to allow easy identification of CRF+ neurons throughout the brain by visualizing TdTomato fluorescence in live tissue. Ex vivo whole-cell electrophysiological recordings from these mice indicate that CRF+ neurons in the CeA have homogenous firing properties to suprathreshold current injections<sup>46</sup>. These neurons appear to be late firing

at the rheobase current, but more closely resemble regular firing neurons at suprathreshold currents. CRF+ neurons also appear to be localized to the CeL in images from coronal brain slices, although an in depth analysis of the distribution of CRF+ neurons within the anatomically defined CeA subregions has not been performed. However, as previously measured by Haubensak et al., CRF+ neurons are largely non-overlapping with PKC $\delta$ + neurons in the CeL. Considering the evidence that CRF signaling within the CeA can generate fear related and anxiety behaviors, and that the vast majority of CRF+ neurons do not co-express PKC $\delta$ , it is possible that CRF+ neurons consist of CeL-On cells (Fig. 1B). Future experiments using optogenetic and chemogenetic manipulations will allow researchers to elucidate the role of these neurons in CeA microcircuitry. For instance, CRF+ neurons may regulate fear and anxiety through inhibition of CeL-Off cells (i.e. PKC $\delta$ + neurons), or through long-range projections to brainstem effector nuclei. In either case, CRF+ neurons in the CeA are seemingly positioned for promoting fear and anxiety, and may serve as an important target for the treatment of anxiety related psychiatric disorders.

## Conclusion

The advent of transgenic mouse reporter lines combined with in vivo optogenetic and chemogenetic techniques has allowed researchers to determine the functional class of neurochemically identified neurons in the CeA. These neurochemically defined cell-types fit into the architecture of CeA microcircuitry that is sufficient for the bidirectional control of fear and anxiety related behaviors. Of note, PKC $\delta$  is expressed in neurons that tune down the expression of conditioned fear or anxiety, whereas SOM is expressed in neurons that are capable of acquiring and producing unconditioned and conditioned fear. In addition, CRF expressing neurons have been implicated in the regulation of defensive behaviors including learned fear and anxiety, but direct evidence of the activity of these

neurons and their place within intra-amygdalar circuitry has not been determined. Although input specific regulation of SOM neurons has been demonstrated, further work is needed to identify the specific inputs of CRF and PKC $\delta$ + expressing neurons, and whether specific inputs are capable of regulating redundant or distinguishable defensive behaviors. Similarly, although the output targets of SOM and PKC $\delta$  neurons have been examined in some detail, analyses of the output of CRF+ neurons remains limited. Overall, the rise of new technologies for manipulating neurons in a cell-type and circuit specific manner will glean insights into how populations of neurons in the CeA cooperate to control fear and anxiety.

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# Understanding the Mechanisms of Neuronal Transport and Homeostasis of the Essential Trace Metal and Environmental Toxin Manganese

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## Abstract

Essential trace metals, such as iron, copper, and zinc, are a biological necessity required to support the structure and function in multitudes of enzymes and other proteins throughout the body. The juxtaposition of a metal's biological necessity and toxicity creates a demand for regulation- through an intricate network of metallochaperone proteins, metal transporters, signaling pathways, and machinery to sequester excess metal in organelles. Manganese (Mn) is another such essential metal, required as a cofactor for diverse set of enzymes including arginase, Mn-superoxide dismutase, and glutamine synthetase. However, little is known regarding intracellular Mn homeostasis. While a nutritional deficiency to Mn has not been described, it is likely that Mn, like other essential metals must be carefully regulated to ensure proper health. The high levels of Mn that occur normally in brain argue for a particular role of Mn in brain physiology and function. For those at risk to occupational overexposure to Mn, the understanding of Mn homeostasis is an important step to preventing or reversing Mn toxicity. This toxicity is characterized by a significant accumulation of Mn in the globus pallidus of the brain, leading to symptoms of "manganism"- a disease resembling the cognitive, motor, and emotional deficits seen in Parkinson's disease<sup>1,2</sup>. Current studies implicate a connection between neurological motor disorders (i.e. Huntington's disease and Parkinson's disease) and unbalanced Mn concentrations in the brain. This review will describe the current knowledge of Mn-handling in cells and neurons in particular.

## Influences of Mn in the Brain

The normal, physiological concentration of Mn in the human brain is estimated to be 5.32–14.03 ng Mn/mg protein (20.0–52.8  $\mu$ M Mn), while 15.96–42.09ng Mn/mg protein (60.1–158.4  $\mu$ M Mn) is the estimated pathophysiological threshold<sup>3</sup>. A variety of factors may affect Mn accumulation and distribution, thereby altering Mn homeostasis and toxicity. In a study of chromium (VI) stress, a two-fold increase in brain Mn levels accompanied increased Cr concentrations<sup>4</sup>. Metabolic stress may alter Mn distribution in tissues, as suggested by a recent study which found decreased levels of Mn in the brain stem and frontal lobe after strenuous exercise relative to control conditions and moderate exercise<sup>5</sup>. Dietary iron levels may have an impact on levels of Mn accumulation in the brain<sup>6</sup>. Ceruloplasmin, a plasma protein involved in the oxidation and mobilization of iron, may also affect the distribution of Mn in brain tissues<sup>7</sup>. These studies point to related mechanisms of Mn and Fe homeostasis.

Mn levels in the human brain have been found to be highest in the putamen, caudate nucleus, and globus pallidus and lowest in the pons and medulla. Human Mn brain levels, especially in the putamen, globus pallidus, and middle temporal gyrus, were found to positively correlate with age. Magnetic resonance and x-ray fluorescence have indicated significant accumulation of Mn in the hippocampus, brain stem and midbrain, basal ganglia, and thalamus as well as the choroid plexus and olfactory bulbs following subchronic Mn exposure<sup>10-12</sup>. On the sub-cellular level, Mn has long been thought to accumulate primarily in brain mitochondria, from which it has been shown to efflux very slowly<sup>13,14</sup>; however, in more recent investigations of intracellular distribution, Mn has been shown to accumulate mainly in the nuclei of cultured choroidal epithelial and brain endothelium cells and in the nuclei and cytoplasm of cultured dopaminergic

in the nuclei and cytoplasm of cultured dopaminergic (DAergic) neuronal cells upon exposure<sup>15</sup>. Furthermore, in neurons and astrocytes of the striatum and globus pallidus, Mn levels were found to be lowest in the mitochondria compared to the cytoplasm, where levels were intermediate, and the heterochromatin and nucleolus, where the highest levels were found. However, in the same study, after chronic Mn treatment the rate of Mn increase was higher in the mitochondria of these cells than in the nuclei, with astrocytes sequestering more Mn than neurons<sup>16</sup>. Sub-cellular distribution of Mn has yet to be indisputably characterized<sup>9</sup>.

Mn speciation and oxidation state may play an important role in its uptake and distribution in the central nervous system, as Mn-citrate has been shown to predominate in the CSF, and Mn<sup>3+</sup> exposures have been shown to result in higher concentrations of Mn in the brain than Mn<sup>2+</sup> exposures<sup>17,18</sup>. Indeed, recently the subcellular dis-

tribution and speciation of Mn within PC12 cells, an immortalized cell line with neural crest origins treated with various Mn compounds, was examined<sup>19</sup>. Differential toxicities and subcellular distributions were observed depending on the chemical form of Mn exposed to the cells. PC12 cells exposed to Mn<sub>2</sub>O<sub>3</sub> demonstrated normal Mn<sup>3+</sup> particles within the cytoplasm with little toxicity, presumably due to its insolubility. For cells treated with MnCl<sub>2</sub>, MnSO<sub>4</sub>, and other organic compounds, Mn<sup>2+</sup> was observed mainly in the Golgi apparatus<sup>19</sup>. The mode of Mn delivery to the brain may mediate patterns of accumulation, as evidenced by the differential distribution across brain regions of injected Mn versus Mn released from peripheral tissues such as the liver<sup>20</sup>. An investigation of low-level Mn exposure via drinking water showed significant levels of Mn deposited in several brain regions including the olfactory bulb, cortex, striatum, globus pallidus, and hippocampus<sup>9, 21</sup>.

## Manganese Exposures

Mn is used for many industrial purposes, including the formation of aluminum alloys, and stainless steel production. Ingestion is the major route for exposure in industrial settings<sup>22-24</sup>, but Mn can also be inhaled. Unlike ingested Mn, inhaled Mn does not pass through the liver and filtered out through a “first pass”, but is directly transported into the brain by the olfactory or trigeminal presynaptic nerve ending<sup>25,26</sup>. In the brain, Mn disrupts dopamine, serotonin, and glutamine signaling<sup>27,28</sup> and can lead to the development of manganism (described above). Workers who are repeatedly exposed to Mn through welding fumes are at risk for manganism/Parkinsonism<sup>23,29</sup>. PARK genes may modulate the DAergic neurotoxicity of Mn-containing welding fumes, as exposure to Mn has been shown to cause mitochondrial dysfunction and alter DAergic PARK protein expression<sup>28</sup>. Mitochondria mediated toxicity is thought to occur in part by interference of ATP activation, leaving an energy deficit<sup>30</sup>. Mn exposure alone promotes apoptosis by the release of caspases and cytochrome c, but in the presence of dopamine, this process is potentiated further. This may in part explain the selective death of DAergic neurons in the striatum following Mn exposure<sup>31</sup>. It also suggests that neuronal apoptosis

is a response to excessive Mn levels in the brain<sup>9</sup>.

*Cellular redox pathways and neuronal oxidative stress.* Production of reactive oxygen species (ROS) following excessive Mn exposure is a canonical response seen in vitro and in vivo<sup>32</sup>. The disabling of antioxidant defenses by Mn that exacerbates this toxicity is also well characterized<sup>13</sup>. Recent work has shown that the toxicity of Mn is largely dependent upon the redox state, as GSH levels can inversely predict toxicity upon Mn exposure<sup>33</sup>. Neuronal cell exposure to Mn induces oxidative damage to DNA, but this can be reversed with glutathione treatment<sup>34</sup>. Antioxidant treatment in chronically exposed rodents reverses not only toxicity but also motor deficits and signaling pathways activated by oxidative stress<sup>35</sup>. The signaling pathways associated with oxidative stress and Mn includes, PI3/Akt35-37 protein kinase C, ERK1/2, p38, and JNK38-40. Interestingly, the phosphorylation of DARPP-32 at Thr34 is induced by Mn, which allows DARPP-32 to act as an inhibitor to protein phosphatase 1 (PP136). The production of ROS is thought to be the cause of nitric oxide synthase and NF-κB induction<sup>41</sup>. The autoxidation of dopamine by Mn is also well documented<sup>42</sup>. MAO activity is in-

crease as a result increased ROS, and this leads to a decrease in dopamine in the striatal cells affected. It has been noted that the depletion of dopamine is independent of the ROS generation, as dopamine depletion has been observed to occur prior to ROS detection<sup>43</sup>. Glutamate uptake by the glutamate/aspartate transporter is inhibited by Mn, leading to higher extracellular glutamate<sup>44</sup>. Expression of GABA transporters are reduced with excess Mn, leading to increased extracellular GABA<sup>9,45-47</sup>.

*ATM-p53 signaling pathway.* Increased levels of p53 have been found in cortical neurons and glial cells from Mn-exposed non-human primates, and analysis of gene expression changes in cortical tissue from these Mn-exposed animals has revealed a prominent role of p53 in Mn-induced alterations in gene expression<sup>48,49</sup>. K-homology splicing regulator protein (KHSRP), a regulatory protein involved in neuronal apoptotic signaling, is upregulated in Mn-exposed striatum along with p53, providing further evidence of the role of p53 in Mn neurotoxicity<sup>50</sup>. Recently, a major p53 response to Mn exposure was found in mouse striatal cells and human neuroprogenitors. Activation of ATM kinase activity was shown to be sensitive to Mn at neurologically relevant concentrations, and inhibitors of ATM kinase decreased Mn-dependent p53 phosphorylation, confirming ATM-p53 as a significant Mn response pathway<sup>9,51</sup>.

*Inflammatory Pathways.* The neurotoxicity mech-

anism of Mn may in part be due to a resulting glial activation and neuro-inflammatory response. Pro-inflammatory cytokines such as IL-6, IL-1 $\beta$  and TNF- $\alpha$  are induced by endotoxins such as lipopolysaccharide (LPS), but potentiated in the presence of Mn<sup>52</sup>. Cytokine toxicity potentiated by Mn has been shown to depend upon the presence of astrocytes<sup>53</sup>. This is thought to be mediated by the activation NF- $\kappa$ B and p38, reflecting how their pharmacological inhibition blocks this effect<sup>54,55</sup>. However, microglia are capable of releasing these cytokines in response to Mn alone<sup>55,56</sup>. Stressing the increased presence of microglia in the basal ganglia relative to any other areas throughout the brain<sup>57</sup>, perhaps this in part can explain the sensitivity of the area to Mn toxicity. Parkinson's disease patients and animal models do demonstrate increased activation of microglia in these areas<sup>9,58</sup>.

It is important to note the damage that continued glial cell activation can have: primarily generating harmful reactive oxygen species (ROS; discussed further below) and reactive nitrogen species (RNS) such as NO, but the overproduction of cytokines can lead to a cascade of further glial activation in the surrounding areas. Recently, it was noted that IL-6 could induce uptake of Mn while also upregulating the Mn-permeable ZIP14 zinc channels, and down-regulating the Mn-exporter SLC30A10<sup>59</sup>. Whether or not this can explain the potentiating effects of Mn with IL-6 and other cytokines is not yet clear<sup>9</sup>.

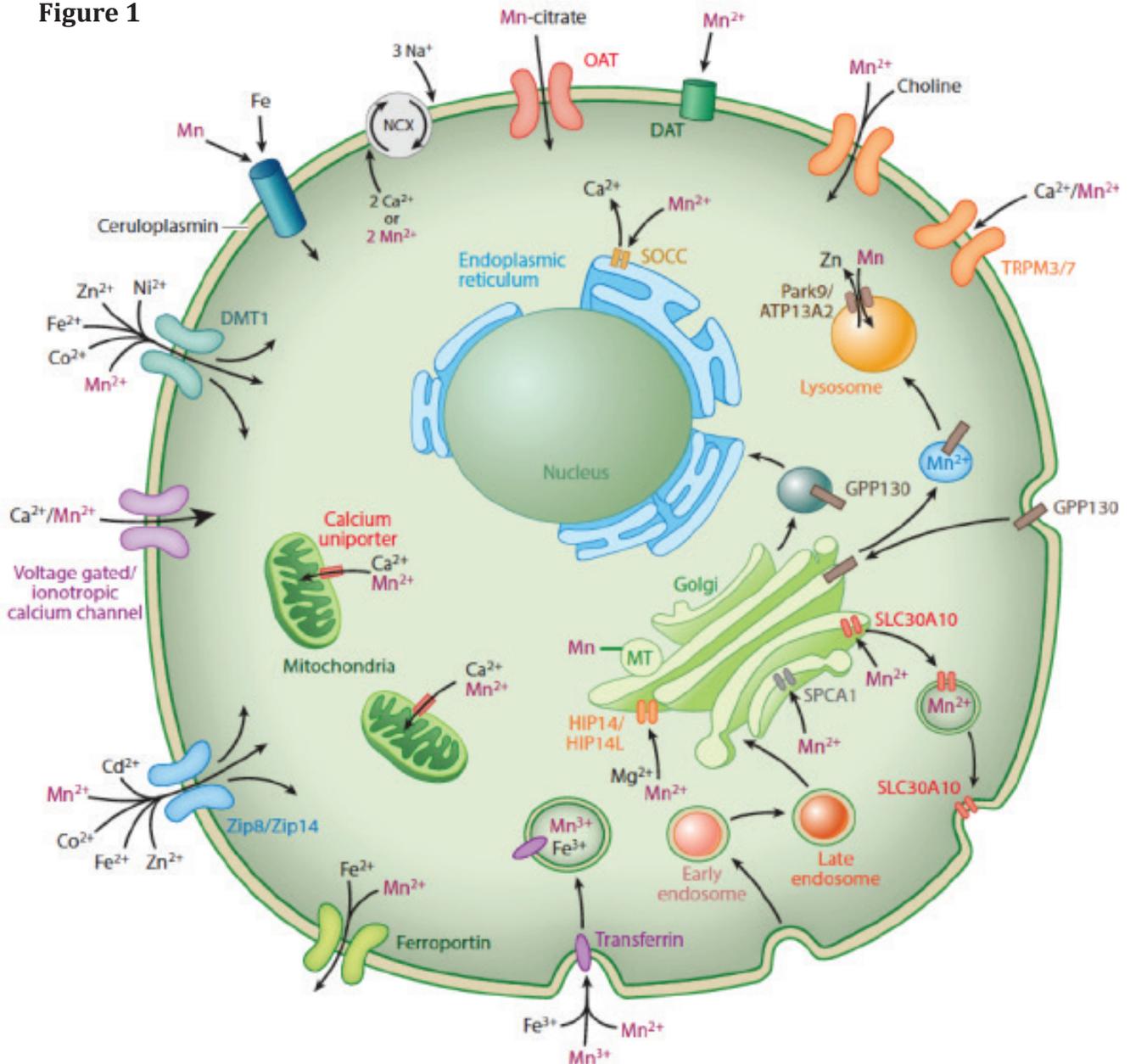
## Cellular Influx of Manganese in the Brain

The mechanisms and details of intracellular Mn transport and storage are under active investigation. Most of the known transporters involved in transport of Mn into and within cells of the brain (including neurons and glia) are non-selective and also transport other essential metals (**Figure 1**). As such, the known Mn transporters cannot explain how intracellular Mn concentrations are selectively maintained without simultaneously strongly influencing the concentrations of other metals. Further, aside from a few notable exceptions (e.g. SPCA1), the manner by which known Mn transporters regulate uptake and efflux of Mn into the cells, versus the subcellular distribution of Mn is not well established. The presumed Mn

transporters and channels are described below.

*The unlikely role of divalent metal transporter 1 (DMT1).* The divalent metal transporter (DMT1) is given its name for its ability to transport several metal cations such as Co<sup>2+</sup>, Fe<sup>2+</sup>, Mn<sup>2+</sup>, and Zn<sup>2+</sup><sup>60,61</sup>. Numerous studies have demonstrated that DMT1 is capable of transporting Mn<sup>2+</sup><sup>44,62-66</sup>, and despite reports of its presence in the BBB<sup>67</sup>, the choroid plexus<sup>66</sup>, and in cells of the basal ganglia<sup>68</sup> where the highest amounts of Mn collect following exposure<sup>69,70</sup>, others still question the role that DMT1 plays in Mn transport in the brain. For example, the pH that is required for Mn to be taken up into cells seems to be different than the pH at

Figure 1



at which DMT1 operates<sup>71</sup> and the mere existence of DMT1 in capillary endothelial cells has also been questioned<sup>69,72</sup>. Despite having less than 1% of functional DMT1, Belgrade rats still have the same concentration of Mn in the brain compared to WT rats<sup>71</sup>. This and other studies<sup>71,73</sup> at least suggest that DMT1 is not the major transporter of Mn in the brain. Recently, Seo and colleagues<sup>74</sup> noted that Mn accumulation increases both in vitro and in vivo neural models following Fe depletion, concurrent with the upregulation of DMT1<sup>74</sup>. An alternative explanation to mediate the conflicting results is that the presence of Fe diminishes the transport of Mn through a receptor independent of DMT1<sup>9</sup>.

*Transport of trivalent Mn by transferrin (Tf).* Although most biological free Mn appears to exist and be transported in its divalent state, a significant portion of Mn is transported as Mn<sup>3+</sup> though a transferrin (Tf) mediated mechanism<sup>75-79</sup>. Much like the case of DMT1, the mechanism by which Tf transports Mn is similar to its normal function of transporting Fe. Though it has been shown that Mn<sup>3+</sup> can still compete with Fe<sup>3+</sup> for Tf transport<sup>78</sup> the former transport occurs at a much slower rate. Despite the similarities of Mn and Fe in their biological activity, these metals differ in their preferred oxidation states, where Fe is much more stable in its trivalent form, and Mn its divalent form. The oxidative potential of Mn<sup>3+</sup> is also stronger

than that of Fe<sup>3+</sup>, so following its deposition by Tf, Mn<sup>3+</sup> may cause unspecified oxidative damage and contribute to Mn toxicity when in excess within the cell<sup>78</sup>. Studies using transferrin-deficient mice note different distributions of Mn in several organs, but no changes from normal Mn concentrations in the brain, suggesting that like DMT1, transferrin is not the primary Mn transporter of the brain<sup>77</sup>. Due to the size of the transferrin complex, Mn or Fe bound to transferrin must be bound to a transferrin receptor and endocytosed in order to cross the plasma membrane. It has been suggested that the transferrin receptor (TfR) works in combination with DMT1 in a mechanism where the pH is lowered in the endosome via V-ATPase, causing the release of Mn from Tf and reduction to Mn<sup>2+</sup>. DMT1 then is able to transport H<sup>+</sup> and Mn in its divalent state into the cytoplasm<sup>9,78</sup>.

Export of Mn by ferroportin (FPN). A third transporter of iron, ferroportin (FPN), allows for the efflux of both Fe and Mn from the cell<sup>80,81</sup>. Mn is capable of inducing FPN mRNA expression in a dose dependent manner<sup>81,82</sup>. Understood as a compensatory mechanism, exposure to Mn or Fe has been shown to change FPN localization in the choroid plexus at the blood-cerebral spinal fluid barrier<sup>66</sup>. Flatiron (ffe/+) mice expressing mutant FPN show reduced intestinal uptake of Mn and Fe<sup>83</sup>, perhaps as a different compensatory mechanism to avoid intracellular cytotoxic accumulation of these ions in the brain. Expression of WT FPN in dopaminergic SH-SY5Y and HEK293T cells is neuroprotective against Mn, whereas expression of mutant FPN in these cells does not bare this neuroprotective effect<sup>83</sup>. Mitchell and colleagues (2014) failed to reproducibly identify a difference of Mn efflux in *Xenopus* oocytes expressing FPN compared to those without FPN expression- finding an actual decrease of efflux of Mn in cells expressing FPN in some cases<sup>84</sup>. It's not understood whether this particular study accounts for the decreased accumulation of Mn in the FPN expressing cells as a reason for decreased efflux, as seen in a previous study using *Xenopus* oocytes<sup>80</sup>. Regardless, the role of FPN as an exporter of Mn has already been recognized in mouse brain *in vivo*<sup>81</sup>, and its association remains much less controversial than a Mn role with DMT1 in the brain<sup>9,85</sup>.

Calcium channels and zinc transporters are also permeable to Mn. Mn has often been used as a tool to observe the functionality of other channels and transporters that traffic divalent ions. For this reason, calcium channels have been identified to be permeable to Mn, often at comparable affinities<sup>86</sup>. Examples of these calcium channels include transient receptor potential cation channels, such as TRPM3<sup>87</sup>, TRPM7<sup>88,89</sup>, and TRPC5<sup>90</sup>. Ionotropic glutamate receptor channels<sup>91</sup>, and store operated calcium channels<sup>92</sup> also have reported permeability to Mn<sup>9</sup>. The permeability of the sodium-calcium exchanger (NCX) to Mn<sup>2+</sup> was first demonstrated in myocardial cells as a surrogate to study Ca<sup>2+</sup> efflux<sup>93,94</sup>. More recently, the inhibition of the NCX channel for 24 hours was shown to increase cellular Mn levels in immortalized mouse striatal neuroprogenitors<sup>51</sup>. Efflux through NCX is a proposed dominant mechanism of Ca<sup>2+</sup> efflux following an action potential<sup>95-97</sup>, however additional studies are needed to determine the role of NCX under normal Mn neuronal homeostatic conditions<sup>9</sup>. The contribution of any these channels to normal Mn transport, storage and homeostasis has hardly been assessed.

A couple of zinc transporters have been implicated in the regulation of Mn in cells. ZIP8 and ZIP14 are most closely related, both acting as divalent ion/HCO<sub>3</sub><sup>-</sup> symporters that drive metals across a HCO<sub>3</sub><sup>-</sup> gradient<sup>98</sup>. When expressed in HEK 293T cells or *Xenopus* oocytes, they are capable of transporting several divalent ions such as Co, Fe, Cd, and Zn<sup>99</sup>. The transport activities of Mn through these two proteins are not negligible, but their affinities are significantly lower than the aforementioned metals. Models studying ZIP8 and ZIP14 have had inconsistent results regarding the magnitude of Mn transport. Nevertheless, rat basophilic leukemia RBL-2H3 cells grown to be Mn-resistant show marked suppression of ZIP8 expression<sup>100</sup>. Knockdown of ZIP14 has shown reduction of Mn uptake in SH-SY5Y<sup>59</sup>. Stimulating inflammatory conditions with IL-6 stimulates the uptake of Mn while concurrently upregulating ZIP14 and downregulating SLC30A10<sup>9</sup>. These studies demonstrate the ability of ZIP family transporter expression to modulate intracellular Mn, however the lack of specificity of these transport-

ers means that cells are unlikely able to change ZIP expression to specifically respond to Mn.

*Other entryways of Mn into the cell.* Crossgrove and colleagues (2003) found that Mn citrate was able to cross the BBB at rates much faster than predicted for diffusion<sup>101</sup>. This suggests that a mechanism exists, at the very least in situ, for transport of Mn bound to citrate across the BBB and plasma membrane. The rates of Mn citrate transport were also significantly faster than Mn alone, indicating that it may be a major mechanism of Mn transport<sup>101</sup>, and suggested to be facilitated perhaps through the organic ion transporter or the monocarboxylate transporter<sup>102</sup>. Suwalsky & Sotomayor<sup>103</sup> noted that exposure of Mn-citrate to the erythrocyte membrane induces far less structural damage than ionic Mn alone, arguably due to citrate's metal-chelating abilities. For this reason, and the large availability of citrate in serum compared to Mn<sup>17</sup>, it would be plausible that citrate is a reasonable source of Mn for the cell. However, the reality of citrate playing a meaningful role in Mn transport has not yet been further tested.

Another possible yet unconfirmed significant route of Mn entrance into the brain is through the choline transporter. Exposure to Mn has been

shown to inhibit choline uptake in perfused rodent brain by nearly 50% within in situ preparation<sup>104</sup>. More recently, Bagga & Patel<sup>105</sup> reported that chronic Mn exposure in mice was associated with decreased levels of choline in the hypothalamus and thalamus<sup>105</sup>. These areas were also marked by a reduction in glutamate, N-acetyl aspartate and N-acetyl aspartate. GABAergic disruption was only damaged in the basal ganglia<sup>9</sup>.

Lastly, considering the numerous connections of Mn with Parkinsonian disorders (not discussed here), it is not surprising that Mn interacts with and is possibly transported by the dopamine transporter (DAT). Based on the observations that chronic exposure to Mn produces PD-like symptoms but spares the DAergic cells of the substantia nigra<sup>106</sup>, it has been suggested that the mechanism would likely be acting at the presynaptic terminal, deactivating DAT<sup>107</sup>. The amphetamine-induced release of dopamine is indeed prevented by Mn<sup>108</sup>. It has also been observed that the presence of Mn induces the internalization of DAT in transfected HEK cells<sup>9,109,110</sup>. Choline, citrate, and dopamine transporters seem like reasonable targets to explore for Mn homeostasis, but fundamental research is lacking.

## Cellular Efflux and Intracellular Transport of Manganese in the Brain

*The Mn-specific exporter SLC30A10.* The only known selective cell-surface transporter of Mn identified as SLC30A10<sup>111</sup> is the strongest evidence for Mn-specific homeostasis so far. Originally described as a zinc transporter based on its family classification, analysis of its amino acid structure distinguishes SLC30A10 from other zinc transporters<sup>112</sup>. Immunohistochemical staining has localized SLC30A10 to the plasma membrane and also throughout the secretory pathway- including the Golgi system and endosomes<sup>113,114</sup>. Transfections of the human SLC30A10 gene into Mn-sensitive yeast cells reversed the obstructed growth phenotype when exposed to Mn. Consistent with the support of SLC30A10 as a Mn exporter, inducing mutations into this gene reverted the cells back to their original Mn-sensitive phenotype<sup>115</sup>. Similar studies in *C. elegans*, HeLa cells, and primary cul-

tures of mouse midbrain neurons have shown that expression of SLC30A10 yields protection from toxic Mn concentrations, and this effect is reversed when the gene is mutated<sup>9,111</sup>. Human patients with mutations in the SLC30A10 share symptoms with manganese patients- including hypermanganesemia, dystonia, cirrhosis, motor neuropathy, and behavioral disturbances with differing severity<sup>113,114,116</sup>. Although SLC30A10 has been shown to transport Zn and other cations other than Mn, it is remarkable that patients with mutations in SLC30A10 do not exhibit changes in concentrations in any other trace metals tested so far in the brain.

*The Mn-specific detection via SPCA1/GPP130.* Another intracellular Mn transporter of great interest, SPCA1, is a Ca<sup>2+</sup>/Mn<sup>2+</sup> ATPase expressed highly in the brain on the surface of the Golgi mem-

brane that transports cytosolic Mn<sup>2+</sup> and Ca<sup>2+</sup> into the Golgi lumen<sup>117,118</sup>. By this mechanism, Ca<sup>2+</sup> can be stored safely, and excess Mn<sup>2+</sup> can be removed from the cytosol and exported through the secretory pathway. SPCA1 can transport one Mn<sup>2+</sup> or Ca<sup>2+</sup> ion at a time per hydrolyzed ATP. Considering its high affinity to Mn, comparable only equally to Ca<sup>119,120</sup>, SPCA1 is recognized as one of only two critical regulators of Mn known to date- the other being SLC30A10. Loss of function in the SPCA1 yeast homologue PMR1, leads to hypersensitivity to Mn toxicity<sup>121</sup>. In humans, the specificity to Mn is even higher than for the yeast protein<sup>122</sup>. The null mutant of SPCA1 in mice is lethal, with heterozygous mice having increased rates of apoptosis and demonstrating larger Golgi with diminished leaflets<sup>123</sup>. Rats exposed to chronic MnCl<sub>2</sub> (30mg/kg i.p. daily for 30 days) had twofold increased expression of SPCA in the mitochondrial proteome in the brain<sup>124</sup>, in what can be assumed as a compensatory detoxification process. However higher concentrations of Mn<sup>2+</sup> exposure (1mM) in cultured mouse neurons and glia have been shown to inhibit Ca<sup>2+</sup> ATPase activity of SPCA1 to approximately 50% of vehicle without influencing expression<sup>125</sup>. The failure to see changes in SPCA1 expression are likely due to a timing difference (30 days as compared to 6 hours) or in vivo/vitro differences, but what can be observed is that SPCA1 can be oversaturated by Mn and toxicity will result from presumably blocking normal Ca<sup>2+</sup> sequestration. On a systems level, a significant amount of detoxification of Mn<sup>2+</sup> may occur in the liver. Knockdown of SPCA1 in HEK293T cells limited growth and decreased viability following Mn<sup>2+</sup> exposure<sup>126</sup>. Overexpression of SPCA1 in these cells allowed for increased Mn<sup>2+</sup> tolerance. Similarly, a mutation to increase the pore size of SPCA1 in yeast resulted in a hyperactive transporter with

increased Mn<sup>2+</sup> efflux and Mn<sup>2+</sup> tolerance<sup>9,127</sup>. Expression of SPCA1 has been identified in neuronal, astroglial, ependymal, oligodendroglial, but not microglial cells<sup>117</sup>. The subcellular distribution of SPCA1 is predominantly reported in the Golgi<sup>128-132</sup>, though the exact subsection is uncertain. Inexplicably, the amount of SPCA1 is not correlated with the amount of Golgi present in the cells<sup>9,133</sup>. The important discovery that Mn exposure induces the cis-Golgi glycoprotein GPP130 to traffic from the Golgi to multivesicular bodies and then to lysosomes for degradation<sup>134</sup> has defined a molecular and biological sensor for Mn that may be involved in Mn homeostatic regulation. The normal function of GPP130 appears to involve the trafficking of vesicle directly from the endosomes to the Golgi bypassing late endosomes and pre-lysosomes<sup>135</sup>. Its sensitivity to Mn is delicate and specific to Mn rather than other metals. This mechanism has been recorded in neuronal cell lines and the degradation of GPP130 from Mn exposure has been demonstrated in vivo as well<sup>136</sup>. Recently, Tewari and colleagues<sup>137</sup> helped to elucidate this sorting mechanism by discovering that GPP130 binds to Mn, inducing oligomerization of the protein. SPCA1 is required for Mn<sup>2+</sup> to reach the Golgi lumen and bind to GPP130, which provides a putative mechanism by which cells regulate excess cytosolic Mn. Increased cytosolic Mn may be pumped through SPCA1 to the Golgi lumen, where it binds to GPP130, induces its oligomerization, resulting in the sorting to the oligomer and secretion of Mn from the cell. Importantly, the degradation of GPP130 is the first and only Mn-specific reporter observed to-date. The degradation of GPP130 can serve as an important biomarker and sensor of changes in intracellular Mn in future experiments, and can lead to the discovery of downstream/parallel signaling pathways regulating Mn<sup>9</sup>.

## Future Directions

With the contributions of major iron transporters (e.g. DMT1, ferroportin) already sufficiently studied, future studies should focus on viable but largely untested candidate transporters and channels, such as the citrate-mediated transport though the organic anion transporter, or via the choline transporter. A few other candidates in-

clude HIP14, HIP14L, and ATP13A2. Before their recognition as transporters for Mg<sup>2+</sup> and other divalent metals like Mn<sup>2+</sup><sup>138,139</sup>, HIP14 and HIP14L were recognized as a required protein for the proper palmitoylation and thus proper cellular distribution of huntingtin protein (Htt) and several synaptic localized proteins<sup>140-144</sup>. Down-reg-

ulation of HIP14 leads to increased inclusions in cells expressing either wild-type or mutant Htt<sup>144</sup>. Considering the connections between HD and altered Mn biology (not discussed here), a putative transporter of Mn that has an imperative role in proper Huntingtin trafficking and proper response to Huntingtin is a compelling link.

ATP13A2, also known as Park9, is a P-type ATPase primarily found in the neurons of the substantia nigra and is a putative cation shuttle across the lysosomal membranes<sup>145-148</sup>. The evidence supporting Park9 transport of Mn comes from studies showing that deletion of the yeast homolog, Ypk9, yields sensitivity to toxicity of heavy metals including Mn<sup>147</sup>. Similarly, a protective effect from Mn is seen when overexpressed in mammalian cell lines or rat primary cell cultures<sup>148</sup>. The mechanism behind the protective effect of ATP13A2 is not understood, but it has been proposed to help sequester toxic metals into vacuoles, or function as Zn/Mn pump as described by Kong<sup>149</sup>.

Metallothioneins (MT) have been well described as a mediator of Zn and other metal homeostasis, though the literature on the relationship of Mn and MT is very sparse. Metallothioneins are Golgi-localized low molecular weight proteins that bind metals at the thiol groups of the cysteine rich residues, and moderate their storage and detox-

Mn is essential to human health. Given that Mn is required for a number of physiological functions but toxic at excessive levels, mechanisms of Mn homeostasis are critically important. Exposure to Mn is mainly dietary and occupational. While ingestion is the major route for exposure, Mn can also be inhaled, especially in certain industrial settings. Normally high levels of Mn in the brain suggest that Mn plays a particularly important role in brain physiology and function and argue for the importance of elucidating mechanisms of Mn homeostasis. A number of candidates for cellular Mn uptake have been identified and investigated, including DMT1 and the transferrin system, zinc transporters, as well as citrate, choline, dopamine, and calcium transporters. Likely candi-

dates for cellular efflux of Mn include SLC30A10, the sodium-calcium exchanger (NCX), and ferroportin. Regulation of subcellular distribution and storage has been attributed to Park9/ATP13A2, SPCA1 and GPP130, and metallothioneins<sup>9</sup>.

Other studies should continue to look for unknown transporters and Mn-related mechanisms. New research to generate novel chemical tools to probe mechanisms of Mn transport was performed via a high throughput screening approach<sup>151</sup>. A total of 41 small molecules were identified from a high-throughout screen, capable of significantly increasing or decreasing intracellular Mn content in a concentration dependent manner using a mouse striatal neural cell line<sup>151</sup>. Understanding the targets of these molecules may improve our understanding of cellular and intracellular Mn trafficking and the regulation of Mn homeostasis. A large range of structural diversity suggests these 41 small molecules are working on a variety of mechanisms, leaving the realistic possibility that some of these small molecules are acting on unknown mechanisms of transport and Mn regulation. In line with this thought, Mn-handling is differentially affected in wild type and mutant huntingtin-expressing striatal cells following treatment with several of the distinguished 41 small molecules identified.

## Conclusion

Mn is incorporated into a number of enzymes that are important for brain physiology and function. These include arginase, glutamine synthetase, Mn-SOD, pyruvate carboxylase, and protein serine/threonine phosphatases-1. Mn-responsive pathways have been identified, including the ATM-p53 pathway. Impaired neuronal Mn handling has been observed in HD. Excessive Mn levels are associated with Manganism/PD, but the link between these conditions remains unclear. Impaired Mn

homeostasis may alter the activity of Mn-dependent enzymes and Mn-sensitive pathways, contributing to neurotoxicity and the pathophysiology of neurodegenerative disorders including HD and PD. Further study is needed to clearly define the mechanisms of Mn uptake and distribution in blood and tissues (e.g. ceruloplasmin). Additional studies are needed to clearly characterize the homeostatic mechanisms that regulate Mn and de-

termine which of the transporters described here, if any, play the most significant roles in cellular Mn transport, distribution, and storage. Further research is needed to explain how alterations in neuronal Mn homeostasis affect Mn transporters, Mn-dependent enzymes, and Mn-responsive pathways to contribute to the pathogenesis and progression of these devastating disorders<sup>9</sup>.

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# Group II Metatropic Glutamate Receptors: Implications for the Treatment of Schizophrenia

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## Abstract

Schizophrenia is a complex disorder that is associated with symptoms in positive, negative, and cognitive domains. Accumulating evidence indicates that a disruption in glutamatergic activity in the prefrontal cortex contributes to the pathophysiology of schizophrenia. Specifically, cortical pyramidal neurons seem to be disinhibited and hyperactive in schizophrenia, leading to elevated glutamatergic function. Glutamatergic dysregulation is implicated in the expression of the cognitive symptoms of schizophrenia, for which there remains an unmet need for treatments. Metabotropic glutamate receptors 2 and 3 (mGluR2/3) are release-modulating autoreceptors that have the ability to dampen the increase in extracellular levels of glutamate, and therefore have the potential to normalize dysregulated cortical neurons. Targeting group II mGluRs may be an effective strategy to alleviate cognitive symptoms and restore brain function in schizophrenia.

**Keywords:** *mGluR2/3; schizophrenia; glutamate hypothesis; prefrontal cortex; dendritic spines*

## Introduction

Schizophrenia is a chronic and severe mental disorder that affects nearly 1% of the population worldwide<sup>1,2</sup>. Schizophrenia etiology has a strong genetic component, with concordance rates of nearly 50% in monozygotic twins<sup>1,2</sup>. Symptoms of schizophrenia are categorized into three main classes: positive, negative, and cognitive<sup>2-4</sup>. Positive symptoms are abnormal by their presence, and include hallucinations and delusions. Negative symptoms are abnormal by their absence, and include anhedonia, blunted affect and social withdrawal. Cognitive symptoms are central to the illness, and include deficits in executive function and working memory<sup>1</sup>. Cognitive deficits are relatively stable across time, persist during the remission of positive symptoms<sup>5</sup>, and are predictive of functional outcome<sup>6</sup> and psychosocial integration. However, cognitive symptoms are resistant to all current treatments.

Several anatomical differences have been discovered from post-mortem and in vivo studies examining the pathology of schizophrenia, including reductions in gray matter volume, especially in the medial temporal lobe and the frontal lobe<sup>7</sup>. Additionally, there is a decrease in the cortical thickness

of the dorsolateral prefrontal cortex (dlPFC)<sup>8,9</sup> in patients with schizophrenia. Despite this decrease in cortical thickness in the dlPFC, there is no significant difference in the overall number of neurons in the neocortex of schizophrenic patients compared to controls<sup>10</sup>. While the number of total neurons appears to be the same, there is a significant increase in neuronal density<sup>8,9</sup>. Taken together, these findings led to the development of the neuropil hypothesis, which posits that the decrease in cortical volume in the absence of overt neuronal loss may be related to atrophy of neuropil, which includes axons and dendrites. In line with this hypothesis, one of the most replicated post-mortem findings is a loss of dendritic spines on pyramidal cells (PCs) in the dlPFC<sup>2,11-13</sup>. Notably, convergent data suggests changes in the dlPFC contributes to many of the cognitive symptoms of schizophrenia<sup>14-19</sup>.

In addition to anatomical differences in patients with schizophrenia, there are significant neurochemical changes, including altered dopamine (DA) function. The DA hypothesis<sup>20</sup> has played a dominant role in guiding research into the pathophysiology of schizophrenia, supported by

the finding that DA receptor D2 (D2R) agonists and antagonists exacerbate<sup>21</sup> and suppress<sup>22</sup> symptoms, respectively. The revised DA hypothesis<sup>23</sup> suggests that hyperfunction in the striatum underlies symptoms of psychosis, while cortical DA hypofunction may contribute to the negative and cognitive symptoms. Antipsychotic drugs (APDs), which are D2R antagonists, are the current treatment for schizophrenia. However, the affinity of an APD for D2Rs is not correlated with treatment efficacy in schizophrenia<sup>24</sup>. Additionally, while APDs alleviate the positive symptoms<sup>25</sup>, they have no meaningful effect on negative or cognitive symptoms<sup>26</sup>, and are associated with many dangerous side effects<sup>27</sup>. Thus, although DA may play a role in schizophrenia, the search for disease-causing mechanisms has expanded to include other transmitter systems.

A role for glutamate (Glu) dysfunction in the pathophysiology of schizophrenia developed following reports of altered levels of Glu in the cerebrospinal fluid of patients with schizophrenia, although these findings were ultimately conflicting<sup>28,29</sup>. The Glu hypothesis of schizophrenia gained momentum after reports that ketamine, an antagonist of ionotropic Glu N-methyl-D-aspartate receptors (NMDARs), strikingly reproduced positive, negative, and cognitive symptoms of schizophrenia in healthy subjects<sup>30-35</sup> and exacerbated symptoms in patients with schizophrenia<sup>36,37</sup>. Further, subchron-

ic treatment with phencyclidine (PCP), another NMDAR antagonist, was found to cause dendritic spine loss in both rodents<sup>38</sup> and primates<sup>39</sup>, thus recapitulating anatomical changes in schizophrenia.

Data concerning the Glu hypothesis suggests there is an increase in extracellular levels of Glu, a finding that may explain anatomical and functional changes in schizophrenia. Interestingly, Glu is a critical determinant of dendritic spine number and morphology<sup>40</sup>. Homeostatic levels of Glu are important in maintaining spine stability in that sharp increases in extracellular Glu culminate in retraction of the spine via sharp increases in intra-spinous Ca<sup>2+</sup> levels<sup>41</sup>. Abnormal cortical processing resulting from disrupted Glu signaling and loss of dendritic spines can have widespread ramifications and may be involved in the expression of cognitive deficits in schizophrenia. Modulation of glutamatergic transmission to dampen the increase of extracellular Glu may then prevent or reverse loss of dendritic spines. Further, modifying disrupted Glu signaling could correct the disrupted cortical processing that contributes to the cognitive symptoms of schizophrenia. The following review will examine group II metabotropic Glu receptors (mGluR2/3), which are release-modulating autoreceptors that have emerged as a promising target for correcting dysregulated Glu signaling and for treating schizophrenia.

## The Glutamate Hypothesis: A Disinhibition Model

The Glu hypothesis is fundamentally centered around the finding that agents such as PCP and ketamine induce psychotomimetic responses and impair prefrontal cortex (PFC) function in healthy individuals, recapitulating symptoms of schizophrenia<sup>30,31</sup>. Furthermore, psychotomimetic agents worsen existing symptoms in patients with schizophrenia, suggesting that these agents affect already vulnerable or compromised mechanisms<sup>32-36</sup>. Given that PCP and ketamine antagonize NMDARs<sup>42</sup>, the Glu hypothesis in its simplest form posits NMDAR hypofunction or an increase in glutamatergic tone.

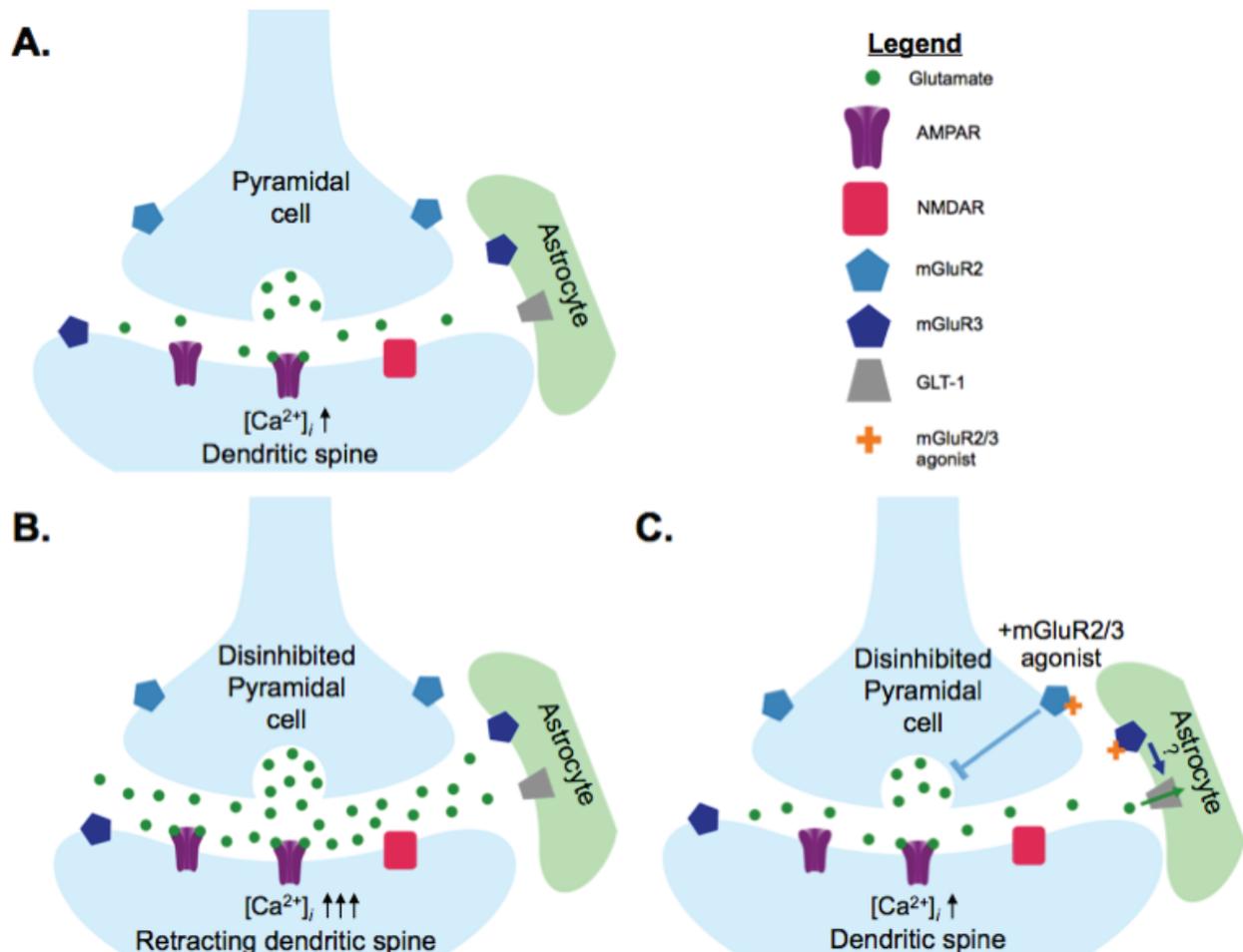
Subsequent findings, however, suggest that the underlying actions and mechanisms of Glu dysfunction are more complex. Studies from Bit-

Moghaddam's group show that treatment with subanesthetic doses of ketamine<sup>43</sup> or PCP<sup>44</sup> lead to an increase in extracellular levels of Glu and DA in the PFC, as assessed by microdialysis in conscious rats. Increased NMDAR antagonist-induced increases of synaptic Glu has been replicated across a number of studies<sup>45-49</sup>. The data suggests that the observed increase in DA following treatment with an NMDAR antagonist is a secondary effect of activation of Glu transmission at non-NMDAR Glu receptors, because treatment with an  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) antagonist attenuated ketamine-induced DA release<sup>43</sup>. Moreover, while both AMPAR and kainate receptor agonists have been shown to increase extracellular levels of DA,

NMDA itself does not<sup>50</sup>. Glu dysfunction, then, may help to explain aspects of DA dysfunction.

In an effort to resolve a mechanism whereby psychotomimetic agents produce an increase of extracellular Glu levels, Homayoun and Moghaddam<sup>51</sup> examined the effect of NMDAR antagonists on fast-spiking GABAergic interneurons, which regulate pyramidal cell activity. They performed extracellular single-unit recordings in the medial PFC of freely moving rats following injection with dizocilpine maleate (MK801), a potent NMDAR antagonist that had previously been shown to produce cortical hyperexcitability<sup>52</sup>. MK801 had a pronounced inhibitory effect on fast-spiking interneurons and, in contrast, a delayed excitatory response in regular spiking neurons<sup>51</sup>. This finding lends support

to a model of disinhibition,<sup>53</sup> in which NMDAR antagonists preferentially target NMDARs on cortical GABAergic interneurons, thereby transiently decreasing GABAergic interneuron firing rates. This decrease in GABAergic firing leads to disinhibition of cortical pyramidal cells, and a subsequent increase in pyramidal cell firing, in Glu release, and in Glu signaling through non-NMDA Glu receptors, specifically AMPARs, resulting in brain circuit disruption (**Fig. 1B**). Importantly, many cognitive functions, including working memory, are dependent upon proper synchronization and spatial tuning of neuronal activity in the PFC<sup>54</sup>. Thus, disruption of glutamatergic transmission and neuronal signaling in the cortex likely contributes to some of the core cognitive deficits in schizophrenia<sup>52,55</sup>.



**Figure 1.** Glutamate hypothesis of schizophrenia: the disinhibition model. Activation of mGluR2/3 may normalize Glu signaling and protect the spine. (A) Under normal conditions, NMDAR activation on GABAergic interneurons (not pictured) provides inhibitory control on cortical neurons to regulate Glu signaling, and moderate levels of synaptic activity and  $[Ca^{2+}]_i$  maintain dendritic spine stability. (B) Hypofunction of NMDARs on GABAergic interneurons results in disinhibition of pyramidal cells, an increase in extracellular Glu, and overactivation of AMPARs.  $[Ca^{2+}]_i$  levels in the spine sharply increase, resulting in spine retraction, and potentially spine loss. (C) mGluR2/3 activation inhibits presynaptic release of Glu and may upregulate the reuptake of Glu from the extracellular space, restoring Glu signaling to near basal levels and therefore regulating  $[Ca^{2+}]_i$  levels in the dendritic spine.

## Overview of mGluRs

A broad understanding of metabotropic Glu receptors (mGluRs) is necessary in order to establish the contribution mGluR2/3 agonists may have in effectively treating aspects of schizophrenia. All mGluRs are G-protein coupled receptors (GPCRs) that are subclassified into three groups based on sequence similarity, G-protein coupling, and ligand selectivity<sup>56</sup>. Receptors within a given group show about 70% sequence identity similarity, while receptors between groups have a sequence identity similarity of approximately 45%<sup>57</sup>. Group I (1 and 5) mGluRs generally couple to phospholipase C (PLC) via Gq coupling and activation. PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to generate inositol 1,4,5-trisphosphate (IP<sub>3</sub>), which releases Ca<sup>2+</sup> from intracellular stores, and diacyl glycerol (DAG), which stimulates PKC. The end result is often depolarization and an increase in excitability. The signaling cascade of group I mGluRs has grown increasingly complex, with the recognition that they can also modulate other pathways<sup>56</sup>.

Group II (2 and 3) and III (4, 6, 7, and 8) mGluRs, conversely, are Gi coupled receptors that inhibit adenylyl cyclase and cyclic AMP (cAMP) formation. Functionally, both of these groups are involved in regulating ion channels, influencing long-term depression (LTD) induction, and contributing to glial-neuronal communication<sup>56,58,59</sup>. Like group I receptors, group II and group III receptors have been found to couple with and modulate additional signaling cascades<sup>56</sup>.

While ionotropic receptors are involved in mediating the majority of neurotransmission, mGluRs are critically important in modulating function at a synaptic level by regulating release of neurotransmitter, influencing other receptors, potentiating or depressing synaptic responses, and more. In this way, mGluRs are prime targets for modulating and restoring glutamatergic transmission. Specifically, group II mGluRs are poised to serve as efficacious targets for treating schizophrenia based on their function, as well as their distribution, which is discussed below.

## Localization of mGluR2/3

Group II mGluRs have unique, but overlapping, expression at both the level of the central nervous system (CNS) and at the level of the synapse. The heterogeneity in their distributions contributes to their value as candidate targets for selectively modulating transmission without large-scale disruption of brain function.

*CNS distribution.* The CNS distributions of mGluR2 and mGluR3 have been extensively examined using immunohistochemistry (IHC) and in situ hybridization<sup>60-63</sup>. The development of more potent and specific mGluR2/3 agonists has resulted in further characterization and quantification of their distribution. Wright et al.<sup>64</sup> utilized a novel, tritiated mGluR2/3 agonist in transgenic mice lacking either mGluR2 or mGluR3 in order to determine the regional quantification of both mGluR3 and mGluR2, respectively. They reported high levels of expression in the PFC and the striatum for both mGluR2 and mGluR3. In the thalamus, mGluR2 dis-

tribution is more restricted to specific nuclei, while mGluR3 is relatively homogenous. mGluR2 and mGluR3 distribution in the hippocampus is highly segregated. While more specific detail outlining the anatomical CNS distributions of both receptors is outside of the scope of this review, it is important to note that the distribution patterns of both receptors indicate distinct and specific roles in glutamatergic transmission, and that both mGluR2 and mGluR3 expression is highly enriched in the forebrain, which is an area of focus in this review.

A major limitation of the field is the lack of antibody specificity for mGluR2 or mGluR3 due to the strong within-group homology, which makes concrete localization of each individual receptor difficult. Therefore, caution must be used in interpreting results from studies that examine protein levels of each receptor separately without use of a transgenic mouse lacking one of the two receptors. Of note, the regional quantitation

of each receptor as assessed by Wright et al.<sup>64</sup> was not wholly additive to total mGluR2/3 levels in a wild type mouse, which suggests there may be compensatory upregulation of one receptor in response to knock out of the other.

*Synaptic localization.* The distributions of mGluR2 and mGluR3 differ from each other on a subcellular level. IHC studies indicate that mGluR2 and mGluR3 stain neuropil, and that they are localized both presynaptically and postsynaptically in the cortex<sup>65,66</sup>. Presynaptically, mGluR2 and mGluR3 are clustered on the preterminal region of glutamatergic neu-

rons, distant from the active zone and in position to be activated by spillover Glu<sup>59,66</sup>. Postsynaptically, mGluR2 is largely found on dendritic shafts and cell bodies, while mGluR3 is located perisynaptically<sup>66</sup>. In both in situ and IHC studies, mGluR3 was also identified in glial cells<sup>60,66</sup>. Recently, Zhang et al.<sup>67</sup> performed RNA-Seq on different purified populations of cells, including neurons and astrocytes. The data from this study suggests that at postnatal day 7, mGluR2 is almost exclusively neuronal with minimal expression in astrocytes. Conversely, mGluR3 is predominantly astrocytic, with comparatively low expression levels in neuronal cells.

## mGluR2/3 Regulation of Extracellular Glu Levels

Homeostatic levels of extracellular Glu are maintained by a complex interplay between mechanisms modulating Glu release, including autoreceptors such as mGluR2/3, and transporters responsible for clearing excess Glu from the synaptic space. mGluR2 is primarily presynaptically located and functions as an autoreceptor, inhibiting further release of neurotransmitters under conditions of high agonist availability<sup>68</sup>. While there is a paucity of data that define clearly the role of astrocytic mGluR3, one possible function is modulation of Glu transporter activity<sup>69,70</sup>.

Activation of mGluR2/3 has repeatedly been shown to decrease the evoked release of Glu. In a striatal synaptosomal preparation, an mGluR2/3 agonist reduced Glu release evoked by 4-aminopyridine by approximately 70%<sup>71</sup>. Battaglia et al.<sup>48</sup> demonstrated the same phenomenon in vivo. Veratridine-evoked release raised synaptic levels of Glu 6 fold in rats, an effect that was completely blocked when rats were pretreated with an mGluR2/3 agonist. Basal levels of Glu were unaffected in animals treated with an mGluR2/3 agonist without evoked activity, a finding that has been replicated across a number of studies<sup>46,72</sup>. Pretreatment with mGluR2/3 agonists also reverse PCP-73 and ketamine-induced<sup>46</sup> in-

creases in extracellular Glu. Direct injection of an mGluR2/3 agonist into the medial PFC inhibited ketamine-induced increase of Glu in this region, suggesting that the actions of mGluR2/3 agonists may take place within the PFC<sup>46</sup>. Together, these findings establish a role for mGluR2/3 in the negative feedback regulation of presynaptic Glu release, and suggest that mGluR2/3 preferentially activates during increased periods of activity and under conditions of high synaptic Glu availability.

Evidence suggests that mGluR3 may regulate synaptic levels of Glu through interactions with astrocytic transporters<sup>69,70</sup>, although the mechanism of interaction remains unclear. Once released from an axon terminal, termination of Glu signaling takes place either through diffusion or by the reuptake of Glu from synaptic space. Astrocytes are critically involved in this process, being responsible for over 90% of Glu uptake in the cortex<sup>74</sup>. Interestingly, Aronica et al.<sup>69</sup> showed that activation of mGluR3 in astrocytic cultures upregulated the expression of two astrocytic Glu transporters, GLT-1 and GLAST. Thus, mGluR3 may serve as an astrocytic rheostat for synaptic Glu and mediate clearance of Glu from extracellular space through interactions with Glu transporters on astrocytes.

## Glutamate Regulation of Spine Morphology

The proposed model of the Glu hypothesis suggests that there is disinhibition of cortical pyrami-

dal cells, contributing to an increase of extracellular Glu and in Glu signaling through non-NMDA Glu

receptors. Excessive Glu signaling is excitotoxic<sup>75</sup>, and can result in damage to, or even retraction of, the postsynaptic recipient: the dendritic spine.

Dendritic spines are highly plastic and compartmentalized postsynaptic structures that can rapidly change morphology in an activity-dependent manner, and contain the biochemical machinery and organization necessary for signaling. Spines are the primary recipient of excitatory transmission to the neuron, and are thought to serve as a neuronal locus for the storage of stable long-term memory<sup>76</sup>. Spines tend to receive a single glutamatergic input; therefore, spine density roughly serves as an index for excitatory drive onto a neuron<sup>77</sup>. Spine elimination is high during development, a time during which neural circuits are refined, and stabilize at a low turnover rate as animals mature<sup>78</sup>.

Spine plasticity is regulated by Glu-mediated activity and a subsequent rise in intra-spinous  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ), an increase that is restricted to the dendritic spine under physiological conditions<sup>79</sup> (**Fig. 1A**). Accumulation of and changes in  $[\text{Ca}^{2+}]_i$  are thought to be a mechanism by which spines are elongated or retract via activation of signaling cascades.  $\text{Ca}^{2+}$  enters the postsynaptic neuron through  $\text{Ca}^{2+}$ -permeable AMPARs, NMDARs, or voltage-gated  $\text{Ca}^{2+}$ -channels. There are also intracellular stores in the smooth endoplasmic reticulum, which are in part regulated by mGluRs via IP3 signaling<sup>80</sup>.  $\text{Ca}^{2+}$  regulates a number of actin-binding proteins, thereby influencing cytoskeletal changes within the spine.

There is an inverse U-shape relationship that exists between the intensity of stimulation a spine

receives and spine morphology<sup>41</sup>. Low-frequency stimulation (LFS) results in low levels of  $[\text{Ca}^{2+}]_i$  and attenuated growth or retraction of the spine. Further, LFS can lead to activation of phosphatases and induce LTD<sup>41</sup>. Moderate levels of synaptic activity resulting in medium levels of  $[\text{Ca}^{2+}]_i$  can lead to growth of the spine and activation of kinases that influence long-term potentiation (LTP)<sup>41</sup>. Both LTP and LTD occurs at the level of an individual spine, as shown by studies that use Glu uncaging to subject single spines to different levels of activity<sup>81-83</sup>. Finally, high levels of synaptic activity resulting in surges of  $[\text{Ca}^{2+}]_i$  culminates in spine retraction (**Fig. 1B**). Excess  $\text{Ca}^{2+}$  activates a variety of phosphatases, proteases, and lipases that result in collapse of the spine.

Changes in a single spine do not have a great impact on the parent dendrite or on the neuron as a whole. This restriction to the dendritic spine is due to the high degree of compartmentalization within a spine. Compartmentalization is thought to be achieved via the spine neck, which acts as a diffusion barrier between the spine and the parent dendrite<sup>76,80</sup>. Therefore, in addition to mediating input-specific plasticity, dendritic spines may also serve as a mechanism for protecting the parent dendrite and neuron. Compartmentalization may help to explain the neuropil hypothesis, which suggests that there is loss of axons and dendrites without overall loss of neurons.

Dampening and buffering excessive Glu stimulation of dendritic spines may prevent the activation of the self-destructive cascade that occurs in response to excess  $[\text{Ca}^{2+}]_i$ , and protect against subsequent spine retraction and loss (**Fig. 1C**).

## Therapeutic Potential of mGluR2/3

Ionotropic receptors appear to be feasible targets to correct disrupted glutamatergic signaling. For example, AMPAR antagonists could be used to reduce the excess glutamatergic transmission that results from pyramidal neuron disinhibition. Clinically, PCP overdose is acutely treated with benzodiazepines, which activate GABAA receptors to markedly enhance GABA-ergic transmission and help restore Glu transmission. How-

ever, ionotropic Glu receptors are expressed in nearly all subtypes of neurons throughout the brain, and are involved in fast, excitatory transmission<sup>84</sup>. GABAA receptors are also broadly distributed throughout the CNS. Pharmacological manipulation of ionotropic receptors could therefore result in profound and broad disruptions in brain function, and have many side effects.

In contrast, targeting mGluRs provides an approach to subtly modulate glutamatergic transmission in a functionally selective manner. mGluR2/3 agonists normalize extracellular Glu levels<sup>44,46</sup>, and thus have the potential to restore Glu signaling (Fig. 1C). mGluR2/3 agonists also attenuate and reverse DA denervation-induced spine loss in the striatum<sup>85</sup>; reverse PCP-induced working memory<sup>73</sup> and motor impairments<sup>73,86</sup>; and attenuate MK801-induced disruption of spike activity and bursting in PFC neurons<sup>87</sup>. Human studies utilizing mGluR2/3 agonists have also demonstrated their potential for treating schizophrenia. Krystal et al.<sup>88</sup> demonstrated that an mGluR2/3 agonist reversed

ketamine-induced cognitive impairments, specifically working memory-related deficits, in healthy human subjects. Further, Patil et al.<sup>89</sup> found that another mGluR2/3 agonist was more effective than the atypical APD olanzapine in treating the positive and negative symptoms of schizophrenia. Treatment with this agonist was not associated with the motor or metabolic side effects that often accompany treatment with APDs<sup>89</sup>. A recent study from Kinon et al.<sup>90</sup> found that patients early in disease showed significant improvement following treatment with an mGluR2/3 agonist, highlighting the potential of mGluR2/3 agonists to curb the rate of the progression of schizophrenia.

## Conclusions

Several lines of evidence suggest that there is an increase in glutamatergic tone in schizophrenia. Because Glu influences dendritic spine number and morphology<sup>41</sup>, abnormal glutamatergic signaling and excess signaling through non-NMDA Glu receptors may lead to changes in spine density, which may in turn contribute to a disruption in cortical processing and the cognitive symptoms of schizophrenia. mGluR2/3 have come to the forefront as promising targets for modulation and correction of glutamate-mediated neurotransmission, and therefore for treatment of cognitive symptoms.

mGluR2/3 are release modulating GPCRs that are distributed throughout the CNS in a heteroge-

neous manner, with distinct regional, cellular, and synaptic-element localizations<sup>91</sup>. These inherent characteristics establish mGluR2/3 as prime candidates for subtle and selective modulation of Glu signaling without overt disruption of brain function. Proof-of-concept studies in both animals and humans highlight the robust potential of targeting mGluR2 and mGluR3 in treating not only the positive symptoms, but also the negative and cognitive symptoms of schizophrenia without adverse side effects. What remains to be determined is whether mGluR2/3 can protect against spine loss in the PFC that results from excess Glu signaling, thereby possibly mitigating PFC-related cognitive deficits.

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## Further Information

Deutch lab website: <http://www.mc.vanderbilt.edu/root/vumc.php?site=deutchlab>

# Cholinergic Modulation of Visual Attention Circuits

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## I. Introduction

We receive details of the external world at an astonishing rate. While sitting at a desk, the amount of sensory information flooding in from the eyes alone is more than can be evaluated at one time. We use a mechanism called attention to focus on certain aspects of our world, at the cost of focusing on other things. As a selection mechanism, attention strongly influences how sensory inputs are processed. The benefit of attending one area over another allows us to perceive with greater contrast and resolution, and is known as spatial attention (Posner 1980; Bashinski and Bacharach 1980; Carrasco 2011). This allocation of processing is also seen on a neurophysiological level (Motter 1993; Desimone and Duncan 1995; Reynolds and Chelazzi 2004). While the neural mechanisms of attention through response enhancement have

been well investigated, there is a largely unexplored role of neuromodulation in this process.

Acetylcholine (ACh) is a neuromodulator proposed to regulate the neural response changes associated with attention and alertness (Sarter et al. 2005). How ACh operates on attention mechanisms in the visual cortex is not clear, although the distinct locations of receptor subtypes suggest several concurrent mechanisms (Disney et al. 2007; Disney et al. 2012). This review will cover how attention alters behavior, modulates neural responses, and may be guided by cholinergic activity in the sensory cortex. While much characterization of cholinergic modulation has already been done in the primary visual cortex of primates, there is a need for expanding this study to associated cortical areas.

## II. Attention

Visual attention has been the subject of scientific inquiry for over a century (Helmholtz 1866). Attention can be deployed through either moving the eyes to a desired location (overt attention), or attending to an area without moving gaze (covert attention). Identical sensory input in the retina can result in different neural activity and percepts due to attentional state, which inspired many studies (Carrasco 2011; Reynolds and Chelazzi 2004; Desimone and Duncan 1995). This has resulted in a wide body of literature connecting neurophysiology with cognition. Paradigms that evoke covert orienting of attention are critical to study the underlying neural mechanisms

### Testing Covert Orienting

The Posner cuing task is an influential paradigm to study visual spatial attention. In the task, subjects respond

to a peripheral target based on a cue (Posner 1980). The subject may attend to the cued location without shifting gaze, which may be valid or invalid in predicting the target correctly. Such a task allows for a direct allocation of attention to one visual field over another. There is a distinct benefit to covertly attending towards the target, and a cost for attending away (Yeshurun and Carrasco 1998, Carrasco et al. 2000; Hermann et al. 2010). This cost and benefit exchange is seen in both changes to reaction time, and in accuracy identifying features of the target. Attention is thought to be a perceptually and physiologically limited resource (Broadbent 1958; Treisman et al. 1960; Reynolds et al. 2000). The biased-competition hypothesis describes this, where stimuli in the visual field compete for processing in a search task. Attending to one stimulus over another biases the activity of neurons processing the selected target's region, while often suppressing activity at the unattended

areas (see: Desimone and Duncan 1995 for review). The signal enhancement hypothesis suggests that attention provides an effective boost in processing in the attended area, at the cost of processing unattended regions (Posner 1980; Bashinski and Bacharach 1980). The representation of stimuli is enhanced at the attended location, including spatial resolution and contrast (Yeshurun and Carrasco 1998; Carrasco et al. 2000; Carrasco et al. 2004; Ling and Carrasco 2006). For stimuli attended away from as a result of invalidly

### III. The Visual Attention Circuit

In order to understand the neural mechanisms behind attention, it is crucial to understand how attentional selection modifies processing in the visual system. Covert orienting has been shown to affect the temporal and spatial properties of visual neuron responses, spanning the prefrontal cortex to the thalamus (Goldberg and Bushnell 1981; Motter 1993; McAlonan et al. 2008). Visually sensitive neurons have receptive fields (RFs), which are regions of sensory space that can alter neural responses (Hartline 1938; Hubel and Wiesel 1968). These RFs can have stimulus preferences with spatial and temporal boundaries. Primary visual cortex neurons show sharper preferences for orientation of gratings, spatial frequency, and ocular dominance, as seen initially in cats (Hubel and Wiesel 1962; De Valois and Tootell 1983). As signals are carried through the visual pathway across the cortex, higher visual areas tend to have larger RFs and more complex tuning dimensions (Van Essen et al. 1992). In the inferotemporal cortex, neurons can be highly selective, responding to specific objects and faces (Tsao and Livingstone 2008). The primary visual cortex (V1) in primates provides a well-described circuit on which attention modulates activity (Levitt et al. 1996; see: Callaway 1998 for review). Furthermore, extrastriate visual areas more commonly studied in attention modulation due to their larger receptive field size, and may present a better alternative.

#### Visual Cortex Organization

The primate early visual cortex is a useful region to examine circuit modulation because the primary projections are well defined (Rockland and Lund 1983; Callaway 1998). Primate V1 receives

cued trials, contrast response functions were attenuated (Pestilli and Carrasco 2005). Neurophysiological data supports this signal enhancement mechanism of attention. Recordings from visually sensitive neurons in primate cortex find a lowered threshold to contrast detection and a sharpening of tuning while the subject is attending to the neuron's receptive field (Reynolds et al. 2000; Reynolds and Desimone 2003; Treue and Martínez-Trujillo 1999; McAdams and Maunsell 1999).

visual input from the lateral geniculate nucleus (LGN) into recipient layer 4c (Fitzpatrick et al. 1994). Spiny stellate cells in layer 4c then send the signals to superficial layers 2-4b, which in turn project to deeper layers 5 and 6, and to extrastriate areas (Callaway 1998). Deeper layers 5 and 6 then provide feedback to layers 2/3 and the LGN, respectively (Fitzpatrick et al. 1994). Hubel and Wiesel identified the visual column structure in the cat primary visual cortex, where they recorded from units along a track running perpendicular to the surface of the cortex. These units shared similar orientation (OR) tuning and ocular dominance preferences (Hubel and Wiesel 1962). Adjacent columns of neurons possessed gradually differing tuning preferences along a continuous gradient, so that a volume of cortical columns may contain neurons with preferences to all orientation values. Later studies have verified that this columnar organization is also present in the primate visual cortex (Hubel and Wiesel 1968). This column of similarly tuned neurons provides a basis for a model cortical microcircuit. In this canonical circuit, inputs arrive in layer 4, and outputs either are feed-forward in supergranular layers 2/3, or feedback through infragranular layers 5 and 6. While details of this microcircuit may change across regions, the fundamental properties provide a general structure for the visual cortex to process information, and provides functional context for recordings across multiple layers (Kohn and Smith 2005).

#### Visual Gain Mechanisms

Neural correlates to attention can take many forms, and have been found across brain regions.

Moran and Desimone first discovered attention modulation of primate visual cortex neurons, which reduced activity to the unattended stimulus in their RF (Moran and Desimone 1985). Further studies have since found that monkeys attending to the locations of a neuron's RF magnify the spike rate of responses in V1 (Ito and Gilbert 1999; Motter 1993); and secondary visual area V2 (Motter 1993), visual area V4 (McAdams and Maunsell 1999; Motter 1993), and medial temporal area MT (Treue and Maunsell 1999). Motter observed changes visual neuron firing rates in response to covert orienting to a bar stimulus, where some neurons were suppressed and others were facilitated (Motter 1993). Furthermore, the tuning curves of V4 and MT neurons were found to scale proportionately with stimulus preference. (McAdams and Maunsell 1999; Treue and Martínez-Trujillo 1999).

Neuron responses to different stimuli contrasts tend to have a nonlinear relationship. This contrast response can be fit by a sigmoidal function to determine the mechanisms of signal gain (Naka and Rushton 1966; Reynolds et al. 2000; Williford and Maunsell 2006; Disney et al. 2007; Soma et al. 2012). Two types of gain control are thought to drive contrast response: contrast gain and response gain. Contrast gain predicts attention modulation to shift the contrast response functions (CRF) towards a lower contrast. This reflects a decrease in contrast required for a neuron to respond, increasing the effective contrast for each stimulus (**Figure 1A**) (Reynolds et al. 2000; Reynolds and Chelazzi 2004). A response gain model of attention predicts an increase in firing rate proportional to stimulus intensity. This reflects an increase in response by multiplying a constant to driving neural rate (**Figure 1B**) (McAdams and Maunsell 1999, Treue and Martínez-Trujillo 1999). These gain control mechanisms are seen as alternative models of atten-

tion (Reynolds and Chelazzi 2004; Reynolds et al. 2000; McAdams Maunsell 1999; Pestilli 2009). Alternatively, some argue for a combination of both models, depending on stimulus and attention field size (Williford and Maunsell 2006; Herrmann et al. 2010). The ability to quantify aspects of visual gain provides insight in how the attention mechanisms may operate. Recent studies involving the neuromodulator acetylcholine have shown similar changes to contrast responses as the response and contrast gain models for attention (Disney et al. 2007; Disney et al 2012; Soma et al 2012).

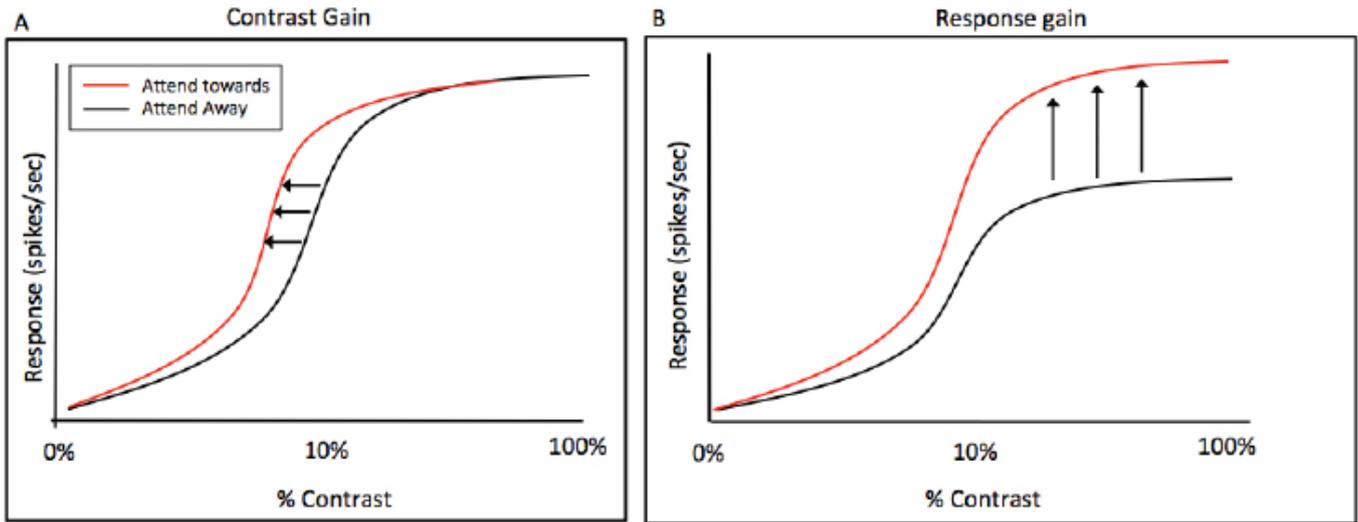
In addition to measuring response modulation by firing rate, there are alternative methods to measure response change by attention mechanisms. Individual neuron responses can be analyzed based on the variability of responses across trials. This can be calculated as the Fano factor, which represents the ratio of firing rate variability to the mean rate (Mitchell et al. 2007; Cohen and Maunsell 2009). Previously, researchers have found an overall decrease in Fano factor of V4 neurons during attended versus unattended cueing (Mitchell et al. 2007). In addition to individual variability measures, there is an increasing focus on group variability of activity across local populations of neurons. These studies examine the influence of attention on a wider scale may be even more important than mean firing rate or Fano factor. Cohen and Maunsell analyzed paired responses of neurons in V4 and found that attention modulation was predicted by reduced in group variability (Cohen and Maunsell 2009). This effect was modeled to have a much higher contribution to attentional modulation than Fano factor or firing rate changes. When comparing neural correlated of attention to behavioral measurements, population responses involving variability may more adequately describe the role of acetylcholine in attention mechanisms.

## IV. Acetylcholine

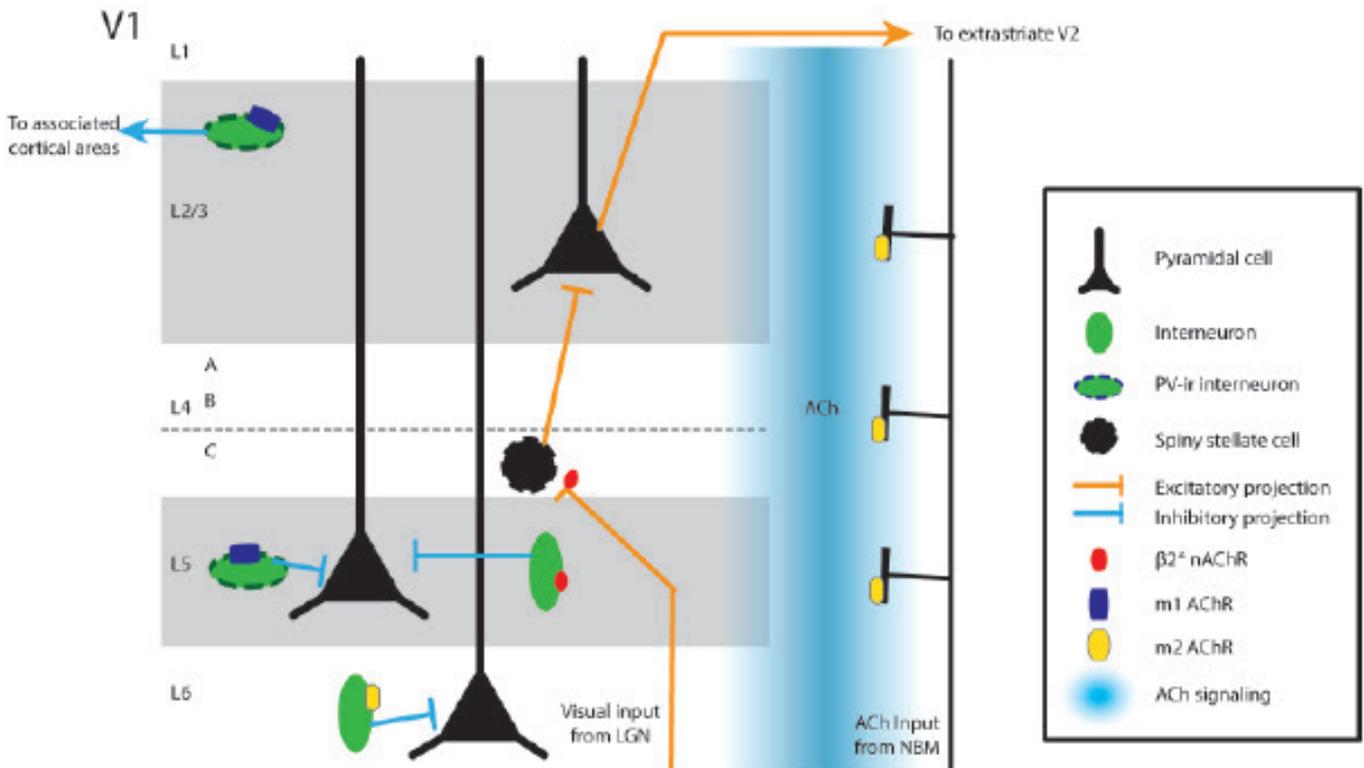
Acetylcholine (ACh) is a regulating molecule in the central nervous system, thought to play a major role in many cognitive mechanisms, as well as sleep/wake cycles. Cortical ACh comes from the basal forebrain, where cortical cholinergic neurons reside (Mesulam et al. 1983). Studies have implicat-

ed cholinergic signaling with attention processes, and have found physiological and behavioral correlates between cholinergic signaling and cue detection (Sarter et al. 2005). Measurements of phasic ACh levels in prefrontal cortex of rats appear to correlate with the spatial attention in a task in-

Figure 1



**Fig 1.** A. Example of contrast gain. A shift to the left in contrast response reduces the contrast required for a neuron to respond. This increases the effective contrast for each stimulus. B. Example of response gain. Neural response is increased proportional to the contrast value. Contrast sensitivity is increased in both cases.



**Figure 2.** Schematic of cholinergic modulation in primate V1.  $\beta_2^*$  nAChRs are present primarily on the presynaptic terminal of the thalamocortical synapse in layer 4C. m1 AChRs are expressed on GABAergic interneurons, including almost all PV-ir neurons. m2 AChRs are present on interneurons, and also act as an autoreceptor on the cholinergic input from NBM. ACh is introduced to all layers from the NBM via volume transmission. Terms: ACh, Acetylcholine; NBM, Nucleus Basalis of Meynert, LGN, Lateral Geniculate Nucleus, V1, Primary visual cortex, PV-ir, parvalbumin immunoreactive.

task involving cue detection (Parikh et al. 2007). Selective lesioning of the basal forebrain leads to attention deficits in monkeys (Voytko et al. 1994). Within the basal forebrain, the nucleus basalis of Meynert (NBM) provides almost all cholinergic innervation to the neocortex (Mesulam et al. 1983). Lesioning the NBM in cats resulted in reduced responses in visual cortex, suggesting that ACh contributes to visual responses (Sato et al. 1987). With widespread projections spanning the cortex, cholinergic afferents signal diffusely to influence regions of tissue.

### Volume Transmission

Afferent projections from the NBM are thought to use non-synaptic volume transmission as their primary form of interneuronal communication. Volume transmission was initially proposed as a method for serotonin and norepinephrine in the cortex (Descarries et al. 1975, 1977). This research demonstrated that neuron outputs often did not form traditional synaptic junctions with an accompanying postsynaptic neuron, and that neurotransmitters can be released non-synaptically into the greater extracellular space (Descarries 1998). Later identified in cortical ACh systems, it is suggested that basal ACh levels are maintained throughout the cortex (Mrzljak et al. 1993; Umbriaco et al. 1994; Descarries et al. 1997, Descarries 1998). It is difficult to identify the extent which cholinergic signaling is non-synaptic, and studies in rats have observed synapses for cholinergic neurons that resemble wired communication of other neurotransmitters (Turrini et al. 2002). Volume transmission allows for widespread circuits to receive regulatory signals from input neurons without direct synaptic innervation, while wired transmission may allow for precise cholinergic influence of specific neurons on a potentially faster time scale. The range of ACh delivery methods in the cortex places an emphasis on ACh receptor location to understand cholinergic function.

### Muscarinic Receptors

Muscarinic receptors (mAChRs) are a subclass of acetylcholine receptors. They are metabotropic G protein coupled receptors, and as such, initi-

ate an intracellular signaling cascade that results in downstream effects (Brown 2010). These receptors contain a protein subunit, known as the  $\alpha$  subunit, that determines the primary effects of the receptor when activated. Muscarinic receptors are categorized by two main types of  $\alpha$  subunits in the central nervous system. These fall into two subtypes: the Gq coupled m1-type (m1, m3, and m5 receptors), and the Gi coupled m2-type (m2 and m4 receptors) (Brown 2010). m1 AChRs are the most populous in primate V1, followed by m2 AChRs (Disney et al. 2006).

Muscarinic receptors play an important role in attentional systems in primates, as shown in several *in vivo* studies. Davidson and Marrocco (2000) injected scopolamine, a mAChR antagonist, into the intraparietal cortex of awake behaving macaques and found a profound deficit in attentional task performance by increasing reaction times to valid trials. This suggests that the mAChR mediated activity may play a role in covert orienting. In another study, attentional modulation of firing rates seen in V1 neurons was impaired with the application of scopolamine, but not with mecamylamine, a nicotinic antagonist (Herrero et al. 2008). This suggests a major role of muscarinic receptors in attentional modulation seen in the visual cortex. While the exact functional role of mAChRs in the visual cortex is yet to be determined, mAChRs are thought to aid in lateral inhibition (Gill et al. 1997; Disney et al. 2012). Immunolabeling shows that most mAChRs are located on interneurons in the primate V1 (Disney et al. 2006). The predominance of mAChRs on inhibitory interneurons suggests the muscarinic system facilitates interneuron inhibition of adjacent areas, acting as a filter for feed-forward signals (Gil et al. 1997; Disney et al. 2012).

m1 AChRs are the most abundant AChR subtype in the primate visual cortex, and is implicated in noise suppression mechanisms. When activated, the Gq signaling cascade affects neuron activity by closing potassium cation (K<sup>+</sup>) channels (Brown 2010). This causes a depolarization of the cell, and increases excitability. Previous investigations have found that m1 AChRs are found primarily on the soma and proximal dendrites of inhibitory interneurons in the visual cortex (Disney et al. 2006).

Furthermore, a vast majority of parvalbumin immunoreactive (PV-ir) interneurons contained m1 AChRs (Disney and Aoki 2008). PV-ir interneurons in the primate cortex are associated with chandelier and basket cell morphology, and with lateral projections across the cortex (DeFelipe 1997). This finding supports muscarinic signaling in the visual cortex suppresses lateral connections to associated regions (**Figure 2**) (Soma et al. 2012; Disney and Aoki 2008; Disney et al. 2012).

In contrast to the m1-type receptors, m2-type receptors (including m2 and m4 subunits) are Gi coupled, and therefore have inhibitory effects on a neuron. Gi signaling cascades result in the closing of high voltage activated calcium cation (Ca<sup>2+</sup>) channels in terminals, which reduces transmitter release (Hasselmo and Bower 1992). The cascade also opens G protein-coupled inward rectifying K<sup>+</sup> channels, keeping the neuron hyperpolarized (Brown 2010). m2 receptors are mostly found in the cortex, with m4 AChRs found mostly in the striatum (Levey et al. 1991). m2 AChRs are found on the presynaptic terminal cholinergic neurons, acting as the only cholinergic autoreceptor for feedback inhibition (Brown 2010; Zhang et al. 2002). Acting as an inhibitory mechanism, these receptors are thought to regulate cholinergic signaling by hyperpolarizing the cholinergic afferents following excessive activity (Zhang et al. 2002).

The role of muscarinic ACh receptors in the visual system appears to be primarily inhibiting lateral and intracortical connections through m1 receptors (Gil et al. 1997, Hasselmo and Bower 1992, Hsieh et al. 2000). There are also differences in mAChR populations across cortical regions. While primate V1 has mAChRs on mostly GABAergic interneurons, and only present on 10% of excitatory neurons, extrastriate area V2 has twice as many excitatory neurons expressing mAChRs (Disney et al. 2006). Furthermore, primate extrastriate area MT also shows a change in mAChR localization, with most excitatory neurons expressing mAChRs as well (Disney et al. 2014). This suggests that the role of cholinergic signaling through mAChRs may vary greatly across the visual cortical pathways, and that additional roles of mAChRs may manifest outside of V1.

## Nicotinic Receptors

Nicotinic acetylcholine receptors (nAChRs) are a subclass of ionotropic acetylcholine receptors that are primarily permeable to sodium. This causes a fast depolarization of the cell and facilitates activation. These receptors are pentameric, having five subunits, and the subunit composition determines the properties and subclass of nicotinic receptor (Picciotto et al. 2000). The two main classes of nicotinic receptors found in the mammalian brain are those with at least one  $\beta 2$  subunit ( $\beta 2^*$  receptors), and the homomeric  $\alpha 7$  receptors, which appear to have promising procognitive attributes, and may facilitate synaptic plasticity (Broide and Leslie 1999; Deutsch et al. 2013).

Among the nicotinic receptors are the high affinity  $\beta 2^*$  nAChRs. These receptors have been quantitatively labeled in the primate V1 cortex, and seem to be primarily located on the presynaptic terminal of incoming thalamic projections onto excitatory neurons in input layer 4c (Disney et al. 2007). This suggests that  $\beta 2^*$  nAChRs may boost signals into the visual system by increasing the chances of thalamic vesicle release onto recipient medium spiny neurons in layer 4c, facilitating feed-forward signaling along the visual pathway (Hsieh et al. 2000, Hasselmo and Bower 1994; Gill et al. 1997; Disney et al. 2007). In contrast with muscarinic AChRs,  $\beta 2^*$  nAChRs rarely appear on V1 interneurons (Disney et al. 2007).

The thalamocortical presynaptic location of  $\beta 2^*$  AChRs strongly suggests the ability to enhance incoming signals from the thalamus. In vitro studies in rat brain slices demonstrate that nicotine increases the magnitude of excitatory postsynaptic potentials (EPSPs) for ascending pathways, but have no effect on corticocortical connections (Gil et al. 1997). This points to feed-forward signaling as the primary pathway of nicotinic facilitation. This hypothesis has been tested by iontophoretically applying nicotine into layer 4c of the primate V1. These neurons exhibited a reduced contrast detection threshold as a result of nicotine application (Disney et al. 2007). Changes in contrast response for these neurons resembles a response gain control of signal enhancement,

seen in attention studies (McAdams and Maunsell 1999). Anatomical evidence shows that these thalamocortical projections innervate dendritic spines, suggesting that the target cells in layer 4c are likely excitatory medium spiny neurons (**Figure 2**) (Disney et al. 2007). This finding indicates a facilitatory role for nAChR activity in the excitatory signaling pathway of visual stimuli in V1.

Another class of nicotinic receptor is the homomeric  $\alpha 7$  nAChR. Although the exact location of  $\alpha 7$  receptors in the primate visual cortex is unknown, there are several studies that suggest  $\alpha 7$  receptors may have a powerful role in cortical therapeutics involving mechanisms of attention

## V. Conclusion

Evidence suggests that ACh plays a critical role in attention mechanisms, through complimentary nicotinic and muscarinic receptor mechanisms. Attention is a selective process and the mechanisms behind attentional signal gain appear to utilize the regulatory aspects of diffuse acetylcholine (**Figure 2**). However, large gaps in knowledge of the modulatory mechanism still remain. For instance, a quantitative analysis of  $\alpha 7$  nAChR location in the visual cortex may clarify its role with the higher affinity  $\beta 2^*$  nAChRs. In addition, changes in receptor distribution across the cortex suggest functionally different roles of ACh throughout the visual system. Differences seen already in mAChR proportions across regions suggest significant changes in cholinergic modulation outside of V1. When comparing V1 and V2 mAChRs, excitatory neu-

(Deutsch et al. 2013; Kem 2000; Yang et al. 2013). Autoradiography of primate cortex found  $\alpha 7$  receptor labeling most dense in layer 1, and moderate labeling in layer 4 of V1 (Han et al. 2003).

The overall role on nAChRs in the primary visual cortex is tied to thalamocortical gain in Layer 4c. Although nAChRs are present throughout the cortex, and appear to have an influence on cognitive tasks, there is little description of B2 nAChR localization in the extrastriate cortex, and  $\alpha 7$  nAChRs remain uncharacterized in the primate sensory cortex. Further anatomical studies may elucidate these systems and suggest potential mechanisms of action.

ron expression of mAChRs substantially increases (Disney et al. 2006). Further studies by Disney and colleagues found increases in excitatory neuron expression of mAChRs in extrastriate area MT (Disney et al. 2014). This shift in representation changes the potential modes of action of muscarinic signaling, from primarily inhibitory effects on lateralized cortical connections, to potentially something different. Anatomical structure of V1 is distinct from the rest of extrastriate, and modulation of neural responses by attention is notably less. These anatomical, functional, and cholinergic differences between V1 and extrastriate suggest a potentially different role of cholinergic modulation in attention systems. Future studies should explore this system beyond the primary visual cortex.

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# Neural Stem Cell Heterogeneity within the Ventricular-Subventricular Zone

Gabriella Rushing

## Abstract

For quite some time, the origin and classification of neural stem cells (NSCs) was not well defined. Attempts to categorize NSCs based on their location, function and marker expression have established that these cells are a heterogeneous pool in both the embryonic and adult brain. The discovery and subsequent characterization of adult NSCs has introduced the possibility of using these cells as a source for neuronal and glial replacement following injury or disease. This review describes the identification, characterization and classification of adult NSCs within the primary neurogenic niche, the ventricular-subventricular zone (V-SVZ). In order to understand how one could manipulate NSC developmental programs for therapeutic use, additional work is needed to elucidate how a NSC is programmed and how signals during development are interpreted to determine cell fate. Implications of NSC heterogeneity established in the embryo on their properties in the adult brain will be discussed with a special focus on how these cells may contribute to the formation of brain tumors. Outstanding questions in the field will also be highlighted. As most efforts to investigate NSC biology have been conducted using murine models, this review will focus on mouse NSCs unless otherwise stated.

**Keywords:** *ventricular-subventricular zone, neural stem cells, positional identity, cell fate, brain tumor*

## Introduction

In the developing brain, multipotent neural stem cells (NSCs) are present in the germinal layers of the ventricular (VZ) and subventricular zones (SVZ) that line the lateral ventricles<sup>1-5</sup> and these NSCs give rise to mature neurons and glia. Originally, it was thought that multipotent NSCs existed only during early development with separate progenitor pools giving rise to neurons and glia<sup>6</sup>. However, [3H]-Thymidine incorporation studies in rodents that began in the late 1950s illustrated that cell proliferation continued in distinct regions of the adult brain<sup>7-9</sup>, contradicting previous beliefs that all cells in the adult brain were post-mitotic. The

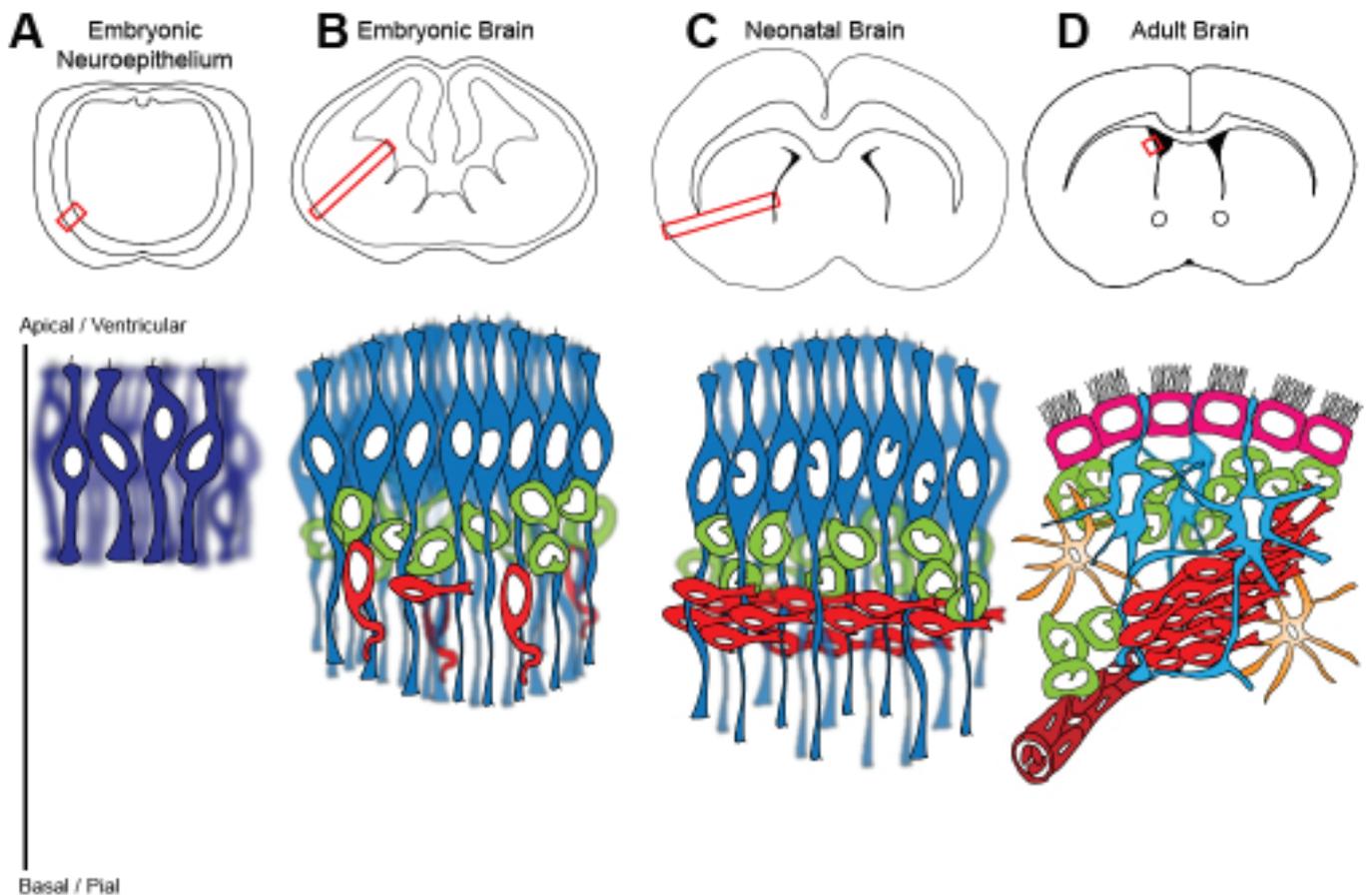
germinal regions identified within the adult mammalian brain were the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus<sup>10-12</sup>. More specifically, these germinal centers have demonstrated continued active neurogenesis and gliogenesis in the adult vertebrate brain of avian, murine and human specimens<sup>13-18</sup>. The focus in this review is on the larger of these two niches, the SVZ, as much work has been conducted highlighting the heterogeneity of NSCs within this niche and their putative contributions to disease states.

## The Neural Stem Cell Continuum: Neuroepithelium to Radial Glia to V-SVZ Astrocytes

*Where do adult NSCs come from?*

The brain develops from a sheet of primary progenitors collectively termed the neuroepithelium, which is derived from the ectoderm. Neuroepithe-

lial cells (NECs) fold in to form the neural plate, which later invaginates to form the neural tube<sup>19</sup> (**Figure 1A**). This transition polarizes the NECs such that the basal side is positioned outward,



**Figure 1.** Development of the Mouse Ventricular-Subventricular Zone. Top= cartoon representations of coronal sections of the mouse brain at distinct developmental stages. Bottom= a cartoon representation of the cells within the red boxed area within each respective coronal plane. Note that the size of coronal sections and corresponding representative images of cell types are not to scale. (A). Neuroepithelial cells (NECs; dark blue) have folded in to form the neural tube. These cells contact both the pial and ventricular surfaces of the developing brain (below) and divide to form a densely packed VZ. (B). In the later developing telencephalon, the NECs give rise to radial glia (RG; light blue), which retain properties of the NECs (see text), including contact with the ventricular and pial surfaces. At this stage, the RG divide asymmetrically, producing a daughter RG and a daughter intermediate progenitor cell (IPC; green) that is pushed away from the ventricular surface, forming a subventricular zone (SVZ). Newborn neurons (red) use the RG processes as a scaffold for migration to their final destinations. (C). In the neonatal brain, the RG are retained until ~postnatal day 7. After postnatal day 2, they begin to retract their basal (pial) processes and will give rise to ependymal (E) cells (pink; shown in (D)), B1 cells (teal; shown in (D)) and B2 cells (yellow; shown in (D)) that remain in the mature brain (D). In the adult brain, B1 cells are the NSCs. They have basal processes that wrap around blood vessels (dark red) and a single primary cilium that traverses between the tightly connected E cells. E cells use their multiple motile cilia to push CSF through the ventricles. Note the presence of transit-amplifying C cells (green), migrating neuroblasts (A cells, red) and parenchymal astrocytes (B2 cells, orange). The structure in the adult is termed the ventricular-subventricular zone (V-SVZ). Note that other cell types exist in these regions that are not discussed within this review including microglia and innervation of the V-SVZ by neighboring neurons.

contacting the pial (outer) surface of the brain and the apical face is oriented inward, which will later become the VZ (See **Figure 1**). Division occurs at the apical surface, forming a ventricular zone (VZ). During cell division, neuroepithelial cells undergo interkinetic nuclear migration (INM), during which the nucleus moves away from the ventricular surface during G1 phase and enters S phase at the top of the VZ then migrates back towards the ventricle during G2 phase and M phase occurs in the VZ<sup>20,21,22</sup>. It is thought that this process serves to move cells in S phase up to allow cell division to occur within the limited space of the VZ (reviewed in<sup>23</sup>). Following neural tube closure, neuroepithelial cells produce specialized bipolar glial cells called radial glia (RG), which serve as the neuronal progenitors in all regions of the CNS<sup>24,25,26</sup> (**Figure 1B**). The transition from NECs to RG has been defined as the acquisition of differentiated glial characteristics including the expression of brain lipid binding protein (BLBP) and glutamate aspartate transporter (GLAST) by neuroepithelial cells<sup>27,28</sup>. Additionally, this transition is characterized by the loss of tight junctions and gain of adherens junctions. RG also express many intermediate filament (IF) proteins including Nestin, Vimentin, and RC2, which have been attributed as markers of NSCs<sup>29</sup> (**Figure 2**). Like their neuroepithelial precursors, RG retain contact with the pial surface of the brain through a basal process as well as maintain ventricular contact through an apical process and they undergo INM during division within the VZ (reviewed in<sup>21,30,31</sup>). During early cortical development, RG divide symmetrically to expand the stem cell population but in later embryonic stages, they divide asymmetrically<sup>30,32</sup> to generate an NSC that persists in the VZ and a daughter intermediate progenitor cell (IPC) that migrates outward to form the subventricular zone (SVZ)<sup>33</sup>. In *Drosophila*, the switch between symmetric and asymmetric

NSC divisions is regulated by Notch signaling<sup>34,35</sup>, where a transient wave of Notch suppression supports asymmetric division. In mice, mammalian partition defective protein 3 (mPar3) regulates this switch: it is dynamically distributed in RG depending on cell cycle progression. Specifically, this distribution of mPar3 leads to asymmetric inheritance of mPar3 by the two daughter cells, resulting in differential Notch signaling such that the daughter cell receiving the greater amount of mPar3 develops high Notch signaling and remains a RG cell while the other (receiving less mPar3) has less Notch signaling and becomes an IPC or a neuron<sup>36</sup>. The IPCs then divide in the SVZ without connection to the ventricular or pial surfaces<sup>30</sup>.

The RG serve dual roles: they act as guides for migrating young neurons, which move long distances along the pial fibers of the RG from the ventricle to the cortical plate<sup>37</sup> and as described above, the RG serve as the progenitors of both neurons and glia embryonically until shortly after birth (**Figure 1C**). Viral targeting and dye labeling via the pial contacting processes of RG using neonatal stereotaxic injections have shown that following postnatal day 2, they retract their processes, lose RC2 expression and generate parenchymal astrocytes<sup>38</sup>, ependymal cells<sup>39</sup> and the adult neural stem cells (NSCs) in the SVZ that continue producing neurons throughout adult life<sup>39-42</sup> (**Figure 1D**). Importantly, transformation of RG into astrocytes has been directly observed with elegant time-lapse microscopy and *in vivo* cell fate analysis experiments<sup>33</sup>. Interestingly, in the postnatal brain most of the VZ compartment is replaced by the ependymal epithelium<sup>43</sup>, thus displacing the primary progenitors in the adult brain from the ventricular surface into the SVZ. Of note, the adult germinal niche includes a subventricular compartment as well as a VZ, resulting in its recent naming as a V-SVZ<sup>44</sup>.

## The Identification of Neural Stem Cells as Astrocytes

The V-SVZ surrounds the lateral ventricles (LVs) and is the larger of the two neurogenic niches in the adult mammalian brain. It is composed of four primary cell types: ependymal cells (E cells), slowly dividing astrocytes (B1 cells), transit amplifying cells (C cells) and neuroblasts (A cells)<sup>45</sup>. Efforts to

elucidate which cell type served as the adult NSC included experiments that infused the antimetabolic drug cytosine- $\gamma$ -D-arabino-furanoside (Ara-C) into the LVs of mice. This resulted in the elimination of all A and C cells with treatment, but B1 and E cells remained<sup>46</sup>. [3H]-thymidine injections in these

mice followed by electron microscopy (EM) analysis showed that most labeled cells following Ara-C cessation corresponded to type B1 cells. Critically, no ependymal cells were labeled, thus identifying V-SVZ astrocytes as the primary precursors for new neurons generated in the adult rodent brain. After cessation of treatment, type C and A cells appear consecutively after 2 and 4 days respectively and the V-SVZ completely regenerates from B1 cells within 14 days<sup>46</sup>. Interestingly, B1 cells exhibited both structural and biological markers of astrocytes, including thick bundles of intermediate filaments positive for glial fibrillary acidic protein (GFAP), a light cytoplasm, glycogen granules, gap junctions and dense bodies<sup>45,47</sup>. Even though earlier reports suggested that E cells served as the NSCs within the adult brain<sup>48</sup>, unlike B cells and their progeny, E cells did not demonstrate DNA synthesis or cell division<sup>46</sup>. This argued against the hypothesis that E cells act as NSCs. The lin-

age of B1 cells within the V-SVZ was further investigated using GFAP-Tva mice, which express avian leucosis virus receptor under the influence of the GFAP promoter<sup>45,49</sup>. Labeling GFAP+ cells by injecting alkaline phosphatase into the V-SVZ of these mice demonstrated that neuroblasts originated from GFAP+ cells, thus illustrating that V-SVZ astrocytes can give rise to new neurons in the adult brain. Additionally, genetic ablation of GFAP+ cells reduced the number of BrdU+ cells within the V-SVZ and diminished the generation of neuroblasts<sup>50,51</sup>. Long-term ablation prevented the production of new neurons, highlighting that the removal of GFAP+ cells destroys the ability of the germinal niche to regenerate itself, which further supports that GFAP+ cells are the multipotent NSCs within the adult brain. Thus, adult V-SVZ NSCs are of glial origin and can be described as being “disguised as astrocytes” (for review, see<sup>52</sup>).

## Architecture of the Primary Germinal Zone

The NSCs found in the adult V-SVZ are kept in a tightly organized spatial niche. The stem cells within this region (type B1 cells) are slowly dividing cells. The ventricular surface neighboring the V-SVZ is lined with multiciliated E cells that create an epithelial monolayer. These cells are responsible for maintaining the flow of cerebrospinal fluid (CSF) through the ventricles of the brain using their multiple motile cilia<sup>53</sup>. While this lining separates the SVZ from the lateral ventricles, type B1 cells (slowly dividing astrocytes/ NSCs) contact the ventricle with an extended process that has a primary cilium, intercalating between the E cells<sup>46,47</sup>. This forms a specific pattern that resembles a pinwheel<sup>43</sup>, with E cells surrounding the apical process of the B1 cell. Their retained ventricular contact via a single apical process mediates exposure of B1 cells to many soluble factors that have the ability to modulate NSC activity<sup>54,55</sup>. Critically, these findings illustrate that B1 apical surface contact is a marker of neurogenic ventricular walls in the adult, as they are absent from the non-neurogenic third ventricle. Interestingly, the apical B1 cell contacts with the ventricle are found to be highly concentrated in three “hot spots”, in the an-

terior-ventral and posterior-dorsal regions of the lateral wall of the LV, as well as in the most anterior portion of the medial wall<sup>43</sup>. B1 cells also contact blood vessels (BVs) through their basal process that terminates in a specialized endfoot<sup>47</sup>. This close interaction with endothelial cells supports proliferation and self-renewal of V-SVZ NSCs<sup>56</sup>. A second subset of B cells referred to as Type B2 cells exists within the V-SVZ, however, they have a multipolar morphology<sup>45</sup> and they are located close to the brain parenchyma and do not have ventricular contact<sup>43</sup>. The role of these cells has yet to be elucidated but it is now known that V-SVZ astrocytes have a dual role in neurogenesis: acting as adult NSCs and as support cells that promote neurogenesis<sup>57</sup>. As B1 cells have been shown to be the slowly dividing adult NSCs, it is likely that B2 cells serve the support role. B1 cells give rise to Type C cells (transit amplifying progenitors) which divide symmetrically three times before becoming migratory neuroblasts (A cells)<sup>58</sup>. Subsequently, the A cells can divide one or two more times on the way to the olfactory bulb (OB). Neuroblasts migrate anteriorly as a network of tangentially oriented chains that converge at the anterior

dorsal subregion of the V-SVZ to form the rostral migratory stream (RMS) <sup>59, 60</sup>. These chains are ensheathed by glial cells, including the GFAP+ basal processes of B1 astrocytes before they terminate on blood vessels <sup>45, 60, 61</sup>. This chain migration has also been observed in young primates and in early postnatal human brain <sup>62-64</sup>.

*What are the destinations of newborn cells postnatally?*

In rodents, the NSCs located within the V-SVZ generate large numbers of neuroblasts that migrate along the RMS into the OB where they differentiate into local interneurons. The function of the OB requires a constant influx of newborn neurons that provide plasticity to the processing of olfactory information <sup>65-67</sup>. Neuroblasts from the V-SVZ mature into granule cells (GCs) or periglomerular cells (PGCs) in the OB. The GCs are all GABAergic interneurons and can be subdivided into types based on the location of their cell bodies after integration: deep, intermediate or superficial layers of the granule cell layer (GCL) and they can be further subdivided by their expression of calretinin (CalR+) <sup>68</sup>. The PGCs can be subdivided into three main non-overlapping subtypes based on marker expression: CalR+, calbindin (CalB+) and tyrosine hydroxylase (TH+) <sup>69</sup>, as well as rare additional cell types <sup>70</sup>. All three PGC subtypes are GABAergic but the functional roles of these cells have not been characterized. In the olfactory bulb, GABAergic inhibitory interneurons greatly outnumber principal neurons by 50-100:1 <sup>71, 72</sup>. The GCs form dendrodendritic reciprocal synapses with mitral and tufted cells, which project their axons to the olfactory cortex to communicate odor information to higher order areas in the forebrain. The large number of inhibitory synapses on the mitral/tufted cells has been suggested to permit inhibitory circuits to refine odor representations <sup>67, 71, 72</sup>. In the adult mouse V-SVZ, it has been estimated that ~10,000 new neurons are generated daily <sup>15</sup>. While a great number of newborn neurons reach the OB daily, roughly half of them are integrated into pre-existing neural circuits while the remaining neurons are eliminated via apoptosis <sup>73</sup>. Whether or not a neuron survives is dependent on the olfactory

sensory experience: sensory deprivation leads to a decrease in new GC neuron survival whereas olfactory learning enhances survival <sup>74, 75</sup>. It is hypothesized that this turnover also contributes to the reorganization of the local OB circuits <sup>66</sup>, allowing for a mechanism of long-term plasticity <sup>76</sup>. This is supported by studies showing that blockade of neurogenesis by administration of an anti-mitotic drug results in a variety of olfaction-related behavioral deficits <sup>77-80</sup>. Thus, a continuous supply of newborn OB interneurons is critical for the plasticity observed in the mouse olfactory system.

Debates regarding the existence of adult-born cortical neurogenesis are ongoing. Many groups have not found supporting evidence in adult murine, primate nor human brain <sup>81-83</sup>, while others have determined that cortical neuron production occurs in the adult mammalian brain but at an extremely low rate <sup>84-89</sup>. Studies reporting ongoing cortical neurogenesis conveyed the presence of adult-born cortical neurons using BrdU incorporation and neuronal markers, which are labeling techniques with limitations. For example, BrdU only labels cells in S-phase, potentially underestimating the number of newborn cells, however, BrdU can also overestimate the number of newborn cells as it can be taken up by neurons undergoing DNA repair <sup>90</sup>. Another important consideration is overlapping marker expression, necessitating staining with multiple markers (reviewed in <sup>91</sup>). Many markers used to identify cells within the NSC lineage are neither static nor unique to progenitor populations- expression patterns often overlap during transition within the lineage. Thus, it is difficult to assess exclusive markers of stem cell populations and experiments require the use of multiple markers to differentiate between cell types. Postnatal gliogenesis has also been reported. Recent work has shown that Nestin+ NSCs in the V-SVZ continue to produce astroglia in the corpus callosum and RMS in adult mice <sup>92</sup>. This is important when considering response to brain injuries, as it has been shown that injury can induce the V-SVZ to produce astrocytes that migrate to the site of damage <sup>93</sup>. Oligodendrocyte production has been observed in the adult corpus callosum and striatum, but are much fewer in number than neuroblasts <sup>94-97</sup>. The pro-

duction of oligodendrocytes postnatally is an important process to investigate, as these cells have

the potential to re-myelinate neurons within the CNS in response to demyelinating lesions <sup>95, 98</sup>.

## Positional Identity of NSCs in the V-SVZ

Recent work has illustrated that cells within the V-SVZ generate specific progeny in the OB postnatally <sup>70, 99-101</sup>. The ability of an NSC to create specific progeny populations based on its location within the V-SVZ is termed a cell's positional identity <sup>42</sup>. For example, ventral NSCs produce mainly deep granule cells (GCs) and CalB+ periglomerular cells (PGCs), while dorsal NSCs mainly produce superficial GCs and TH+ PGCs. Thus, NSCs are not a homogeneous mix of equivalently plastic cells, but rather a spatially organized set of restricted, diverse populations <sup>42, 70, 73, 99, 100</sup>. Importantly, heterotopic transplantation experiments have shown that these cells maintain their positional identity and continue to produce the types of progeny expected from their original location (i.e. cells derived from the ventral V-SVZ transplanted to the dorsal V-SVZ still produced deep GCs and CalB+ PGCs, and not superficial GCs nor TH+ PGCs) <sup>99</sup>. This suggests that identity is mostly a cell-intrinsic feature and that cells "remember" positional cues experienced during the establishment of patterns in the developing brain. Thus, it is likely that there is a progressive restriction of developmental potential. This is highlighted by work investigating cortical development <sup>102</sup>. The mammalian cortex consists of six layers of projection neurons and these neurons are born in an "inside-out" manner such that neurons of the deepest layer are born first and subsequently, the superficial layers are born in a progressive fashion (the outer-most neurons are born last) <sup>103</sup>. Intriguingly, the embryonic NSCs that form these layers become 'restricted' over time. For example, VZ NSCs isolated from older ferret embryos (during the generation of superficial layers) were transplanted to younger embryos (during the generation of deep layers) and only produced neurons in the outer cortical layers <sup>102</sup>, which suggests that the timing of specific neuronal production is also cell-intrinsic. There is evolving evidence to suggest that epigenetic mechanisms are key to maintaining NSC function and potential over time, specifically chromatin-based tran-

scriptional regulation <sup>104, 105</sup>. However, more work is needed to elucidate how a cell's identity is progressively restricted and subsequently maintained postnatally (See Box 1 for additional outstanding questions). While positional identity has been the most characterized in mice, it has also been explored in the marmoset brain using IF analyses of transcription factor (TF) expression. Primarily, these data suggest that there is a spatial heterogeneity, similar to that observed in the murine brain, but that it is only observed in the early postnatal period and the diversity of V-SVZ NSCs may decline with age <sup>106</sup>. It remains to be determined whether positional identity exists in the human V-SVZ and whether primate and human-specific structures are derived from specific progenitors.

### *How is NSC fate encoded?*

Recent work in mouse utilizing time lapse microscopy on the developing cortex has illustrated that the multipotent state of NSCs is correlated with oscillatory expression of several fate-determination factors including the basic helix-loop-helix TFs *Ascl1/Mash1* (neurons), *Hes1* (astrocytes), and *Olig2* (oligodendrocytes), while the differentiated (committed) state correlates with sustained expression of a single factor <sup>107</sup>. This work warrants further exploration into what controls the expression of these factors over a fine time scale and how signaling inputs are integrated to determine the fate of a cell. There is evolving evidence to suggest that epigenetic mechanisms, specifically chromatin-based transcriptional regulation, are critical to maintaining NSC function and potential over time <sup>104, 105</sup>. For example, the developmental switch from neurogenic divisions to gliogenic divisions is regulated in part by DNA methylation: the promoters of astrocytic genes (*GFAP*, *S100β*) possess STAT responsive elements that remain methylated (inactive) until ~E17 when the switch occurs <sup>108</sup>. As time progresses during development, methylation on astrocyte promoters is lost and allows for STAT3 binding and astrocyte production to begin, though

the exact mechanism is not well understood <sup>108, 109</sup>. A recently discovered epigenetic modulator of NSCs is mixed-lineage leukemia 1 (Mll1), a chromatin-modifying factor of the trxB family that activates gene expression <sup>104</sup>. Mll1 is expressed throughout the V-SVZ; when it is deleted, NSCs remain proliferative and are efficient at gliogenesis, but the production of neurons is severely impaired and the neurogenic TF Dlx2 is not expressed. Thus, fate-specifying TFs can be direct targets of chromatin-modifying factors and affect NSC cell fate decisions. Additionally, the long non-coding RNA Pnky has been shown to regulate neurogenesis <sup>110</sup>. Depletion of Pnky results in an increase in commitment to neuronal fates by increasing the tran-

sit amplifying pool and subsequently causing a depletion of V-SVZ NSCs. Still, further investigation is needed to elucidate how these regulatory elements might affect positional identity in the adult

*How is neural stem cell heterogeneity established?*  
A common developmental feature is the regionalization of NSC niches that allows progenitor cells in different regions to become different subtypes of cells. The prime example of this feature is in the developing spinal cord where distinct dorsal-ventral boundaries of transcription factor (TF) expression are generated by gradients of morphogens <sup>111-114</sup>. This coincides with the production of different types of neurons and glia depending on

**Box 1: Persisting Questions**

- Is regional TF expression controlled in the same manner throughout development and in the postnatal brain?
- How is regional identity maintained postnatally?
- Is there a threshold to induce plasticity of NSCs? For example, if we expose cells to certain extrinsic factors, will their developmentally-established program change to produce a different cell type?
- Can we measure inputs on NSCs to determine whether it will make a neuron, astrocyte or oligodendrocyte?

the concentration of morphogen the cell experiences. Regionalization also occurs in the developing telencephalon <sup>115-118</sup>, which later contributes to the formation of the V-SVZ. For example, the lateral ganglionic eminence (LGE) in the developing telencephalon expresses a set of TFs including Dlx1/2 <sup>119</sup>, Mash1 <sup>120</sup>, Gsh2 <sup>100</sup>, Pax6 <sup>121, 122</sup> and Sp8 <sup>123</sup> that are also expressed in the lateral wall of the adult V-SVZ. Furthermore, transplantation experiments in which LGE cells were placed into the adult V-SVZ showed that these cells were able to efficiently migrate to the OB whereas cells from the medial ganglionic eminence (MGE) migrated extensively towards the cortex <sup>124</sup>. Although these cells exhibited unique migratory potentials when transplanted into the adult, Cre-lox fate mapping of the embryonic telencephalic neuroepithelium has since determined that the MGE, LGE and the embryonic cortex all generate NSCs that inhabit different parts of the adult V-SVZ <sup>100</sup>. Adult V-SVZ NSCs (B1 cells) also share this regional specification as shown by the previously mentioned RG targeting experiments <sup>99</sup>. This has implications for therapeutic applications in humans- if the same regional-

ization occurs in human brain, the generation of specific neuronal subtypes following injury may be contingent on whether the region-specific NSCs that produce them are still present in the adult.

Recent evidence has suggested that region-specific TF expression underlies the generation of distinct populations of OB interneurons. For example, a small microdomain of anterior ventral V-SVZ cells that express Nkx6.2 have been shown to generate novel OB interneuron subtypes including deep-projecting GCs, shrub GCs, perimitral cells and satellite cells <sup>70</sup>. Additionally, the homeobox gene Emx1 is expressed primarily in the developing pallium and in dorsal V-SVZ NPCs postnatally <sup>100, 125, 126</sup>. Emx1+ NPCs generate CalR+ superficial GCs and PGC interneurons <sup>126</sup>. Another TF of note is SP8, which is expressed in the developing dorsolateral ganglionic eminence, cortex and septum <sup>123</sup>. SP8+ NPCs mostly produce CalR+ interneurons in the OB. These findings emphasize the link between TF patterning in the developing brain and cell-type specification postnatally. The transcriptional heterogeneity of

these NPCs suggests that they are restricted to specific fates shortly after birth. Future work involving loss- or gain-of-function experiments may

provide information on a putative 'transcriptional code' that determines the types of OB interneurons produced from NPCs in the adult V-SVZ.

## Further Characterization of V-SVZ NSCs

Within adult mammalian stem cell niches, both quiescent (qNSCs) and activated NSCs (aNSCs) exist<sup>127</sup>. Previously, distinguishing between these cells was difficult due to a lack of markers available to identify different stages in the NSC lineage. Specifically, distinguishing between adult NSC astrocytes and niche astrocytes has been a major limitation as both express GFAP<sup>45, 47</sup>. However recent work suggests that B2 cells (parenchymal astrocytes) lack CD133 expression<sup>128</sup>. Doetsch and colleagues have isolated a subpopulation of B1 astrocytes that express epidermal growth factor receptor (EGFR). These correspond to aNSC astrocytes as they are eliminated by antimetabolic treatment and reappear with the first set of dividing cells that regenerate the V-SVZ<sup>129</sup>. Properties of qNSCs within the niche have also been highlighted<sup>128</sup>. qNSCs are CD133+ and Nestin-negative, but upregulate both Nestin and EGFR when activated. Interestingly, Nestin expression is regulated in a cell-cycle-dependent manner during embryonic development<sup>130</sup>. While

Nestin expression has been considered a marker of NSCs in both embryonic and adult brain<sup>131, 132</sup>, these findings highlight that Nestin expression is dynamically regulated and cannot be relied on as a single marker of all NSCs. Mainly, qNSCs are dormant and are negative for the proliferation markers Ki67 and MCM2 that are expressed in actively dividing cells<sup>133</sup>. In contrast, aNSCs (GFAP+, CD133+, EGFR+) have a fast cell cycle<sup>58</sup>. Further characterization has determined the proliferation dynamics of B1, C and A cells<sup>134</sup> using three distinct cell cycle techniques and demonstrated that cell cycle dynamics were similar across different subregions of the V-SVZ. Interestingly, actively dividing B1 cells had a short S phase (4 hours) as compared to C cells (14-17 hours). This progenitor population analysis established that following the initial division of B1 cells, C cells divide three times and A cells only one or two times. This work provides a crucial explanation of how neurogenesis is maintained in the adult brain.

## The Adult Human V-SVZ

The topology of the adult human V-SVZ differs from that of the rodent in that it contains a hypocellular gap that separates the ependymal cells from a periventricular ribbon of astrocytes<sup>135</sup>. This gap is filled with GFAP+ processes containing intermediate filaments and gap junctions. The astrocyte ribbon is present in the lateral ventricular walls, but not in the medial (septal) wall as determined by examination of both intraoperative and autopsied human brain specimens<sup>135</sup>. Isolated cells from the lateral wall had ultrastructural characteristics of astrocytes by electron microscopy, expressed GFAP and vimentin, proliferated *in vivo* and acted as multipotent progenitors *in vitro*. In rodents and primates, newborn neurons (neuroblasts) migrate from the SVZ in chains along the RMS<sup>60, 62, 63, 136</sup>. Interestingly, Sanai and colleagues did not observe any evidence to support that a similar process occurs in adult human brain<sup>135</sup>. A struc-

ture was observed near the origin of the human olfactory peduncle, termed the olfactory trigone, which contained displaced ependymal cavities and GFAP+ processes but no Tuj1+ or PSA-NCAM+ cells (markers of immature neurons). This suggested a lack of chain migration wherein neuronal precursors migrate as individual cells or that migration from the V-SVZ to the OB does not occur in adult humans. It is still unclear to what extent ongoing neurogenesis occurs in adult human brain. Curtis and colleagues reported extensive proliferation within adult human V-SVZ specimens examining tissue from autopsy cases<sup>17</sup>. This contrasts with the observations of Sanai and colleagues who found that less than 1% of SVZ astrocytes were dividing by measuring Ki-67 expression and BrdU incorporation within adult human V-SVZ specimens obtained from intraoperative procedures and autopsies<sup>135</sup>. Perhaps the difference in observations

is that Curtis and colleagues used proliferating cell nuclear antigen (PCNA) as a marker for proliferation while Sanai and colleagues used Ki-67. This highlights that interpretation of studies involving human specimens should be carefully examined and that future studies are needed to determine the degree of neurogenesis in the adult human brain.

*Has the human V-SVZ been examined at different stages of development?*

Studies on the infant V-SVZ have recently been conducted and illustrate that the structure of the infant V-SVZ differs from that observed in the adults. The infant V-SVZ lacks an astrocyte ribbon and a gap layer, but has cells with RG processes expressing GFAP and vimentin that line the lateral ventricular wall, which aligns with what has been observed in human fetal brain<sup>137, 138</sup>. Additionally, a dense network of elongated putative migratory neurons was discovered in the infant V-SVZ. These cells possessed ultrastructural characteristics of young migrating neurons including free ribosomes, microtubule networks and leading processes containing growth cones<sup>60</sup> and expressed the immature neuronal markers DCX, TuJ1 and PSA-NCAM. Human brain specimens ranging from birth to 84 years of age were examined and between 6 and 18 months of age, the V-SVZ progressively loses its network of putative migratory neurons and forms structural characteristics observed in adult human V-SVZ, including an astrocyte ribbon and hypocellular gap layer<sup>64</sup>. Specifically, they discovered an initial robust stream of tangentially migrating immature neurons (DCX+ chains)

populating the space that becomes the postnatal human gap layer, but these chains are gradually decreased between 6 and 18 months of age. In contrast, only a small number of proliferating cells were observed in adolescent and adult specimens. A third study confirmed these findings: they identified an RMS in human fetal brain specimens, but found few DCX+ neuroblasts in the same region when analyzing adult human brain specimens<sup>139</sup>. Thus, these studies suggest a robust period of neurogenesis and neuroblast migration that persists into postnatal human life, but is restricted to early infancy. Another intriguing discovery by Sanai and colleagues was the presence of an additional migratory stream of DCX+ cells that branched off of the proximal limb of the RMS and ended in the ventromedial prefrontal cortex (VMPFC), which they termed the medial migratory stream (MMS). The MMS was observed in 4-6 month specimens, but not in specimens between 8-18 months of age. The MMS appears to be a unique feature of humans as it was not observed in rodent and has not been reported to exist in other vertebrate species. The function of the VMPFC in children has not been elucidated but in the adult human brain, it is activated during certain cognitive tasks including spatial conceptualization and emotional processing of visual cues<sup>140, 141</sup>. Of note, this region is functionally inactivated in patients with advanced Alzheimer's disease<sup>142</sup>, suggesting important functional implications involving processes that change with age. Thus, the MMS is hypothesized to serve as a mechanism of delayed postnatal plasticity<sup>64</sup>.

## Neural Stem Cells, Positional Identity and Their Relation to Brain Tumors

As the adult brain has a limited pool of proliferative cells, it has been hypothesized that neural stem and/or progenitor cells are the cells of origin for many brain tumors<sup>94</sup>. A subpopulation of cells within brain tumors have been identified that possess characteristics of NSCs including self-renewal, multipotency and the expression of similar cell surface receptors (EGFR and PDGFRa)<sup>143-149</sup>. An emerging hypothesis describes these cells as brain tumor stem cells (BTSCs) that are generated from the V-SVZ<sup>149, 150</sup>. Specifically, the V-SVZ

has been proposed to be the origin of gliomas<sup>151, 152</sup>, as many gliomas present in the periventricular region or are contiguous with the V-SVZ. Gliomas associated with the V-SVZ tend to have a worse prognosis<sup>153</sup>, however, it is less clear whether a specific V-SVZ subregion contributes more or less to tumor growth or whether NSCs within the V-SVZ are indeed the cells of origin for these tumors. Despite unclear evidence in glioma tumors, there is emerging evidence that implicates NSC positional identity as an important feature in the gener-

ation of certain tumor types as discussed below.

*Tuberous Sclerosis Complex: An Interesting Case of Location-Specific Tumor Development*

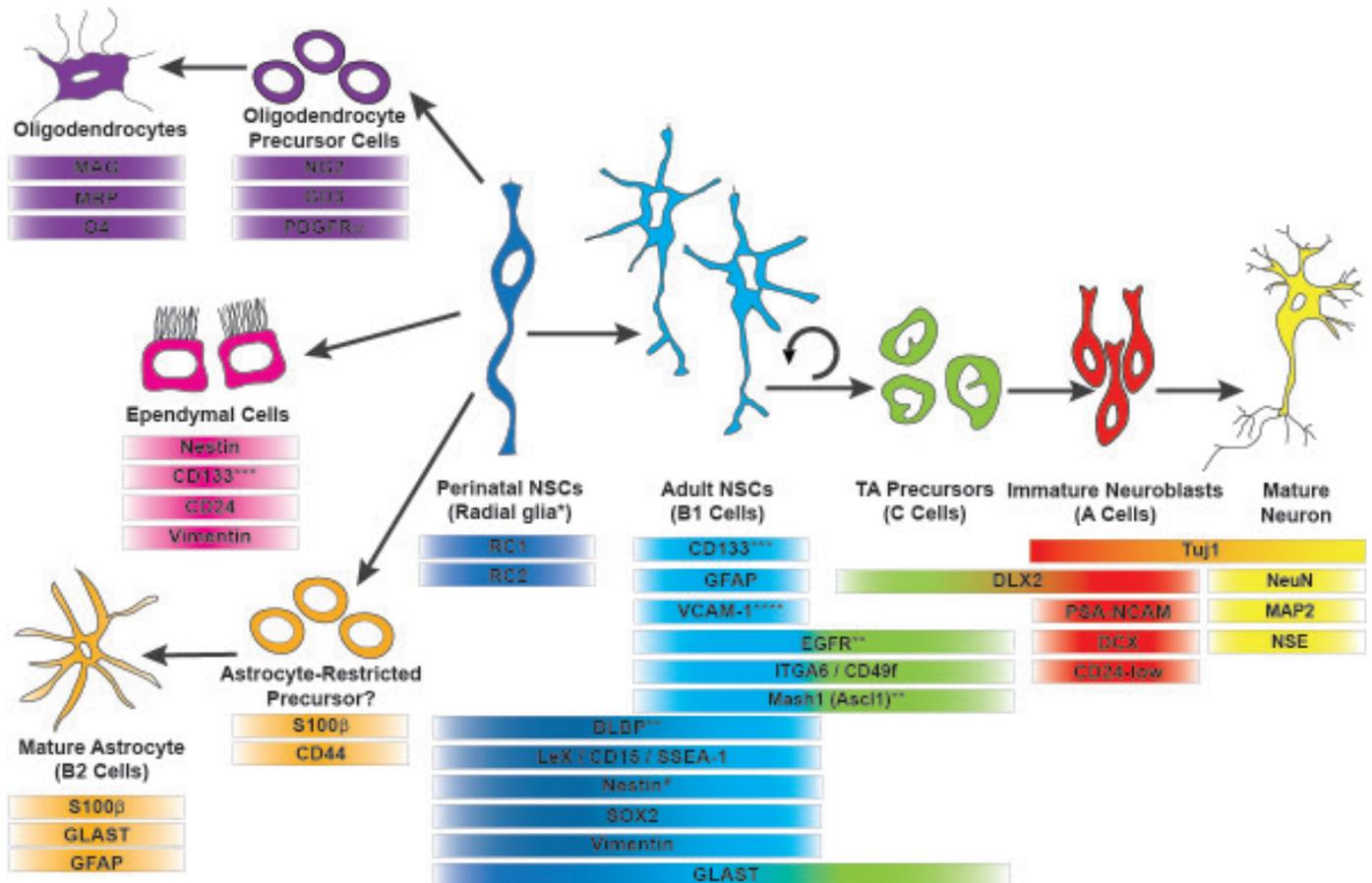
As described, adult neural stem cells are derived from radial glia (RG), which are the stem cells of the developing brain. The RG are thought to be the cells of origin for the tumors that grow in patients with Tuberous Sclerosis Complex (TSC) during development. TSC is an autosomal dominant disorder with a birth incidence of approximately 1 in 6,000 people around the world<sup>154</sup>. It is caused by mutation in either of two genes, TSC1 or TSC2, that encode hamartin and tuberin, respectively (reviewed in<sup>155</sup>). These proteins normally serve as negative regulators of the mammalian target of rapamycin (mTOR) pathway, but upon mutation of either gene, patients exhibit mTOR pathway hyper-activation resulting in significant increases in protein translation, cell size and proliferation (reviewed in<sup>156</sup>). Clinically, the neurological phenotypes of TSC are characterized by the formation of cortical tubers and two types of benign tumors in the brain: subependymal nodules (SENs) and subependymal giant cell astrocytomas (SEGAs). Immunohistochemical analyses on patient samples have revealed that SEGAs express both glial and neuronal markers, suggesting that they develop from the abnormal differentiation of progenitor cells<sup>157</sup>. Both tumor types present in the V-SVZ, but SEGAs are confined to a specific ventral subregion of this neurogenic niche near the foramen of Monro. Part of the pathological criteria for determining whether a tumor is a SEGA is based

on location presentation, with the other being size<sup>158</sup>. SEGAs in the V-SVZ can obstruct the flow of cerebrospinal fluid (CSF) through the lateral ventricles and the increased brain pressure can be lethal if left untreated<sup>159</sup>. It is currently unknown why SEGAs present in the ventral V-SVZ and why some individuals develop these tumors while others do not. Very recent evidence has emerged illustrating consistent nuclear expression of thyroid transcription factor 1 (TTF-1) (also known as NK2 homeobox 1 (Nkx2.1)) in SEGAs examined from 6 patients' neurosurgical specimens<sup>160</sup>. Nkx2.1 is expressed in the embryonic MGE and ventral LGE<sup>161-164</sup> and is maintained in early postnatal and adult brains in the ventral tip of the V-SVZ<sup>165, 166</sup>, which is close to the foramen of Monro where SEGAs present in TSC patients. This leads us to consider whether something may be unique about the cells within the ventral V-SVZ that causes them to be more susceptible to TSC mutations? Is there something special about the ventral V-SVZ niche that impacts proliferation and maturation of the RG leading to SEGA formation? These, among other questions, require further investigation. Exploring the connection between subregions of NSCs and the location of tumor formation is of use when considering the development of novel, focused therapeutic options for patients with TSC or other brain tumors. For example, if SEGAs truly originate from a regionally specified cell-of-origin, potential therapeutics targeting the specific cell lineage could be developed that would avoid negatively affecting other cell types in the region.

## Conclusion

Ongoing adult neurogenesis in the mammalian brain has been a subject of widespread debate from the initial identification of postnatal proliferation to the cell types responsible and the extent to which it occurs across different species. Characterizing NSC functions and features holds promise in revealing mechanisms of cellular programming and determinants of cell fate from a developmental biology perspective as well as in

guiding therapies for many diseases with neurological phenotypes. Although proliferative potential is very restricted in the mature brain, the NSCs are part of complex niches under the control of a variety of cell-intrinsic and extrinsic features that may reveal avenues for therapeutic intervention and thus, further examination of their regulation and potential is warranted.



**Figure 2.** Postnatal Neural Cell Lineage and Marker Expression Profiles. \*Radial glia persist only during the first postnatal week and are non-self-renewing during this time. They retract their processes after postnatal day 2 in the mouse and give rise to parenchymal astrocytes (orange), ependymal cells (pink), oligodendrocytes (purple) and astrocyte-like adult neural stem cells (B1 cells, teal). B1 cells are self-renewing and give rise to transit amplifying progenitors (green), which in turn produce neuroblasts (red) that will mature into neurons (yellow). \*\*These markers are primarily expressed by activated B1 cells. \*\*\*CD133 is present on the primary cilia of B1 cells, as well as ependymal cells. \*\*\*\*VCAM-1 is expressed on quiescent B1 cells. #-Note that Nestin is not expressed on all RG and B1 cells but rather, is dynamically regulated (see text).

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# Oscillator Dysfunction in Autism Spectrum Disorder

*David Simon*

## Abstract

Autism Spectrum Disorder (ASD) is a highly prevalent developmental disability characterized by deficits in social communication and interaction, restricted interests, and repetitive behaviors. Recently, differences in sensory and perceptual function have gained an increased level of recognition as an important feature of ASD that contributes to lifelong disability. These sensory and perceptual differences are believed to result from a specific impairment in the ability to integrate information across neural systems. A crucial mechanism in this integrative process is the rhythmic synchronization of excitability across neural populations, which is known as oscillation. Harmonic oscillation is believed to form the mechanistic basis of efficient and flexible recruitment of functional networks. These functional networks then form the foundation of large-scale cognitive operations including perception. In ASD there is believed to be a global deficit in the ability to organize networks using oscillations. Reductions in the ability to synchronize networks appropriately during sensory and perceptual tasks have been found in a number of frequency ranges. The hierarchical organization of oscillatory activity also appears to be disrupted in ASD, and this structuring is believed to play a critical role in perception. This review discusses evidence that the integrity of oscillatory synchronization in ASD is disrupted, and how disturbance of this neural mechanism gives rise to alterations in sensory and perceptual function. The clinical implications of impaired neural synchronization and future directions for oscillatory synchronization research in ASD are also discussed.

## Introduction

Autism Spectrum Disorder (ASD) is a developmental disability characterized by persistent deficits in social communication and interaction, and restricted or repetitive interests<sup>1</sup>. An estimated 1 in 68 children born in the United States<sup>2</sup> will receive a diagnosis of ASD, and the disorder carries enormous social and economic costs<sup>3, 4</sup>. This high prevalence and socioeconomic cost have motivated numerous investigations into the neural pathology of ASD. Studies utilizing functional magnetic resonance imaging (fMRI) have consistently indicated that patterns of structural<sup>5</sup> and functional<sup>6</sup> connectivity are significantly altered in individuals with ASD. Investigations emphasizing connectivity on more rapid time scales utilizing electroencephalography (EEG)<sup>7</sup> and magnetoencephalography (MEG)<sup>8</sup> have similarly indicated that connectivity is altered. These connectivity alterations have been proposed as both a leading biomarker and the origin of dysfunction in the dis-

order<sup>9</sup>. The emergence of graph theory for quantification of networks has indicated that, despite the heterogeneous nature of connectivity differences among individuals<sup>10</sup>, a consistent pattern of altered organization emerges. Individuals with ASD have networks with reduced long range connectivity<sup>6, 11</sup>, increased short range connectivity<sup>12</sup>, and decreased network modularity<sup>13</sup>. How these changes in network structure impact transient neural processing and emerge as the behavioral ASD phenotype has become an area of important investigation. Studies using EEG and MEG to examine such processing have uncovered differences in rhythmically modulated networks known as oscillators. This oscillatory dysfunction in ASD forms the bridge between basic neurobiology, large-scale network changes, and the sensory and perceptual processing differences that have been increasingly recognized as core features of the disorder.

## Sensory and Perceptual Function in ASD

In addition to the core features of ASD, alterations in sensory and perceptual processing have long been recognized<sup>14</sup>, and revisions to diagnostic criteria have recently acknowledged sensory dysfunction as a core feature of ASD1. Investigations of sensory function in ASD have revealed that, even within a single sensory modality such as vision, both deficits and advantages are present. For example, individuals with ASD consistently outperform their typically developing (TD) peers in terms of accuracy and response speed in visual search tasks<sup>15,16</sup>, and similarly excel at visuospatial tasks<sup>17</sup>. In other visual tasks, such as discrimination of visual motion<sup>18</sup> or gestalt perception<sup>19</sup>, they have significant deficits. This pattern is also present in other sensory modalities; individuals with ASD excel at detection of pitch change<sup>20,21</sup> but are impaired in the ability to utilize gaps in noise to assist with speech comprehension<sup>22</sup>. Tactile discrimination thresholds may also be superior in ASD<sup>23</sup>, although this is more debated<sup>24</sup> and may have a level of stimulus and location specificity<sup>25</sup>. This inconsistency in performance, with both enhancement and impairment of abilities, defines sensory and perceptual function as an area of difference rather than deficit.

An account of perceptual differences that has gained increasing support is that individuals with ASD have deficits in perceptual integration, the process of combining disparate sensory representations. In other words, individuals with ASD possess normal or even superior processing of stimulus characteristics but fail to combine sensory information appropriately<sup>26</sup>. Tasks such as

motion discrimination require integration of localized evidence and are impaired by this deficit, despite their simplistic sensory composition. In contrast, visual search of complex stimuli does not require this process and may be impaired by integrative processes that compete with direct comparisons based on stimulus characteristics<sup>27</sup>. This hypothesis receives support from experimental manipulations of the perceptual complexity of visual stimuli. In individuals with ASD performance continuously degrades as the need for feature integration increases<sup>28</sup>. The presence of both enhanced and impaired processing is also notably absent when tasks require the processing of inputs from multiple senses. In these multisensory tasks, individuals with ASD reveal deficits regardless of the level of perceptual complexity<sup>29</sup>. The level of impairment in ASD further rises with the increased need for perceptual integration<sup>30</sup>. The weak central coherence (WCC) model<sup>31</sup> of ASD is particularly consistent with this pattern of sensory performance. WCC proposes that differences in ASD are based in deficits of information integration across cognitive mechanisms while localized processing remains intact<sup>31</sup>. From a neural perspective, WCC proposes that mechanisms of information transfer and interaction between cognitive systems are impaired on timescales relevant to perception. Investigators have increasingly turned to non-invasive neuroimaging and neurophysiological techniques to investigate these differences. These investigations have uncovered that harmonic neural synchronization is altered in ASD<sup>32</sup>.

## Oscillations and the Senses

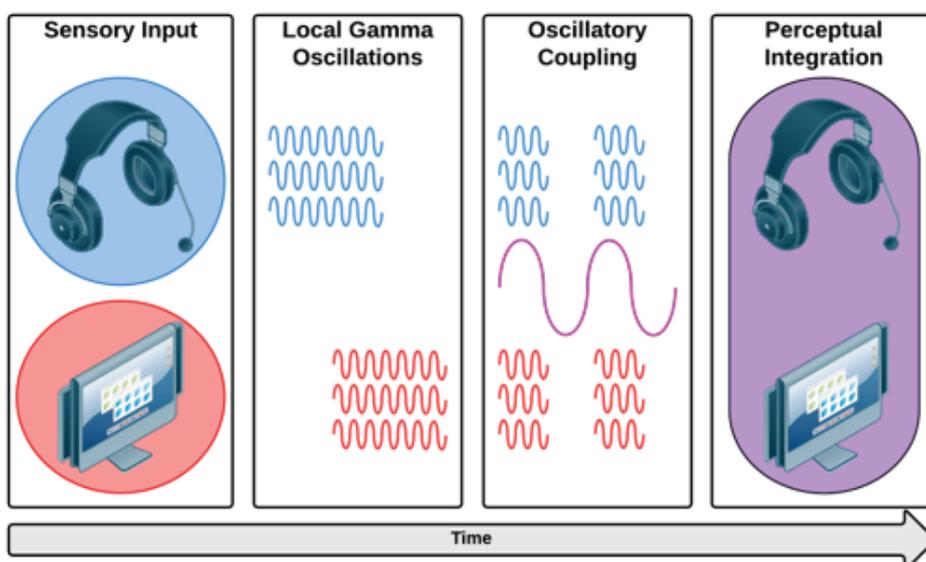
The rhythmic nature of neural activity has been recognized since the earliest attempts at non-invasive measurement<sup>33</sup>. These fluctuations are referred to as oscillations. The quantification of oscillations has led to the recognition that they occur over a large range of frequencies (here denoted as delta:  $\delta$ , 1-4 Hz, theta:  $\theta$ , 4-8 Hz, alpha:  $\alpha$ , 8-14 Hz, beta:  $\beta$ , 15-30Hz, and gamma:  $\gamma$ , >30Hz, although the exact ranges vary in the literature). The functional role of these oscillations in neural computa-

tion is of great interest and has motivated studies designed to establish their origin. At the cellular level, these studies have indicated that oscillations originate in fluctuations of the local field potential, and are generated primarily by synchronized postsynaptic activity in pyramidal neurons<sup>34</sup>. These studies have also found that neurons have properties that facilitate synchronization, such as intrinsic resonance<sup>35,36</sup> and a mixture of predictable harmonic and responsive relaxation proper-

ties<sup>37</sup>. At the circuit level, this harmonic synchronization appears to be an optimized mechanism of network organization, allowing modulation of responses and synchronization of outputs at a low energetic cost<sup>38</sup>. The optimal nature of synchronization is also supported by modelling studies in the field of network science, which indicate that forming small world networks<sup>39</sup> through harmonization is more efficient<sup>40</sup> and flexible<sup>41</sup> than direct connections. Neurons participating in these synchronized assemblies experience temporally aligned fluctuations in membrane potential that track the observed oscillatory phase<sup>42</sup>. This synchronized phasic modulation of neuronal excitability represents an effective method of encoding information and shaping network interactions.

Due to their recognized ability to shape network activity, oscillations have been proposed to play a critical role in both sensory processing and perception. This functional role is supported by the influence of both pre-stimulus oscillatory power and phase on the perception of sensory stimuli<sup>43-47</sup>. At the neural level, evoked responses to sensory inputs also depend on oscillatory state<sup>48, 49</sup>. These studies establish that baseline (pre-stimulus) synchronization plays an important role in processing and perception. An example of these effects is that the phase of  $\alpha$  band oscillations in visual cortices contributes to determining whether near threshold visual stimuli are perceived<sup>45</sup>. These interactions are also reciprocal, in that phase locked oscillations can be observed in evoked responses

and contribute to subsequent perceptual processing<sup>47</sup>. This reciprocity establishes that synchronization is an inherent property of sensory processing, and that disruption of this process could lead to altered sensory function. In addition to these influences, oscillators have also been proposed to play an important role in perceptual integration. Particularly relevant to the discussion of perceptual disruptions in ASD is the hypothesis that coupling of sensory networks facilitates the merging of sensory information into percepts<sup>50</sup>. Support for this hypothesis can be found in the observation of oscillatory hierarchies, in which  $\delta$ ,  $\theta$ , and  $\alpha$  oscillations constrain and organize activity in the  $\beta$  and  $\gamma$  bands<sup>51-53</sup>. In this model of perception, localized networks contain representations of sensory evidence in the  $\gamma$  band. These networks are then unified by oscillatory coupling at lower frequencies<sup>54</sup>. This larger integrated network then forms the basis of perception and contributes to behavior<sup>55</sup>. A simplified version of oscillatory coupling and perceptual integration for two sensory modalities is illustrated in **Fig. 1**. A disruption in synchronization at either high or low frequencies would impact the process of integrating information into percepts in this model. This would be consistent with the deficits in perceptual function observed in ASD<sup>56</sup> and proposed by WCC<sup>31</sup>. Investigations of synchronization at both high and low frequencies in ASD, and the correspondence of disruptions with altered perception, have demonstrated that just such a relationship exists.

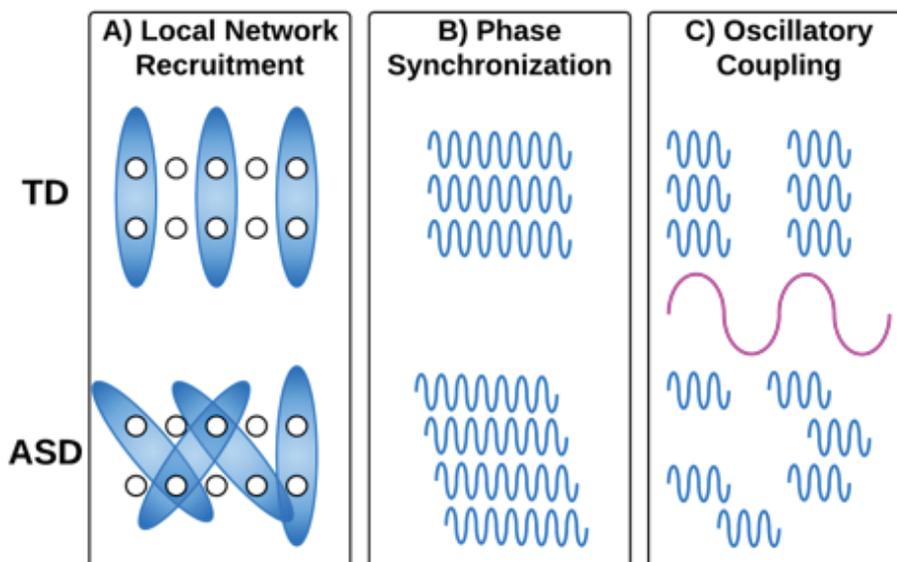


**Figure 1)** Simplified oscillatory hierarchy. Auditory and visual inputs result in localized gamma oscillations that encode sensory evidence. Gamma oscillations are then organized by lower frequency oscillations. Perceptual Integration results from synchronization of component networks.

## Alpha Abnormalities in Sensory and Perceptual Processing

Alpha is one of the most distinct frequency ranges in human neural activity, notable for its significant deviation from the expected  $1/F$  relationship between frequency and power<sup>38</sup>. This atypical power distribution implies functional significance, which has been demonstrated in numerous studies linking  $\alpha$  to sensory and perceptual processing of visual inputs<sup>57-60</sup>. The ongoing phase of  $\alpha$  has been similarly linked to auditory<sup>46</sup> and tactile perception<sup>44</sup>, and interregional  $\alpha$  phase synchronization has been shown to contribute to multisensory perception<sup>61</sup>. Due to this putative importance,  $\alpha$  has become a primary target of investigation in ASD, and these studies have indicated the presence of alterations in both  $\alpha$  power and phase locking. Some of the most direct evidence of  $\alpha$  dysfunction in ASD arises from tasks involving perceptual judgments of visual stimuli. In a task requiring discrimination of sinusoidal gratings from a zebra, TD individuals demonstrate increasing induced (non-phase locked)  $\alpha$  power in visual cortices with increasing levels of spatial frequency. In individuals with ASD, the correspondence between  $\alpha$  power and spatial frequency is still present, but the overall magnitude of power modulation is significant-

ly reduced<sup>62</sup>. Furthermore, the initial peak in  $\alpha$  power occurs earlier in ASD<sup>62</sup>. This has been interpreted as an overall reduction in the level of specialization in the neural networks recruited during this task (**Fig. 2A**). Further analysis indicated that the overall consistency of evoked (phase locked)  $\alpha$  activity in the ASD group was also lower in this task<sup>63</sup>. This decreased consistency across trials also supports the notion of decreased network specialization as it indicates recruitment was less consistent in ASD. An important point, however, is that altered  $\alpha$  in this study did not correspond with differences in reaction time or discrimination accuracy. Further evidence of altered  $\alpha$  recruitment in ASD visual processing can be found using photic driving, in which intermittent light pulses are used to entrain a corresponding neural frequency and influence processing of later stimuli<sup>47</sup>. Photic driving has revealed that the ability to phase lock  $\alpha$  and  $\beta$  to repetitive stimulation is reduced in ASD<sup>64,65</sup>. These studies lead to the important conclusion that individuals with ASD are less able to synchronize neural assemblies at moderate frequencies in response to sensory inputs.



**Figure 2)** Types of oscillator disruption found in ASD. A) Recruited Networks are less specialized and more variable. B) Phase synchronization is reduced. C) Disorganized oscillatory coupling.

Anticipatory modulation of  $\alpha$  power in response to sensory task demands appears to be disrupted in ASD as well. Unlike altered stimulus driven synchronization, these differences have also been robustly linked with behavioral deficits. When stimuli are presented in multiple sensory modalities

and only one is relevant based on a previous cue, selective attention is used to suppress the irrelevant stimulus. Typically,  $\alpha$  power is suppressed in regions that will process the relevant stimulus and power is increased in areas that will process the irrelevant stimulus<sup>66,67</sup>. Children with ASD fail to

appropriately modulate  $\alpha$  power when performing this task with competing auditory and visual stimuli<sup>68</sup>. Furthermore, on trials where a distracting stimulus is present, they show significant performance impairment<sup>68</sup>. This study shows that the ability to suppress synchronization in anticipation of task demands is impaired in ASD, and that this has significant perceptual consequences. Inappropriate attentional selection has previously been theorized to underlie many ASD traits<sup>69</sup>, and this

study demonstrates that impaired synchronization contributes to inappropriate attentional selection. Evidence of reduced stimulus driven  $\alpha$  synchronization and excessive task based  $\alpha$  synchronization may appear to be inconsistent. Both, however, can be characterized by an inability to modify synchronization away from the baseline state. This indicates that the ability to consistently recruit networks to meet cognitive demands is reduced in ASD.

## Gamma Abnormalities in Sensory and Perceptual Processing

Much like  $\alpha$ ,  $\gamma$  is modulated as part of sensory evoked responses and has significant importance to sensory and perceptual process. Importantly, the physiological origins of  $\gamma$  oscillations are also more concretely known; high  $\gamma$  (>80 Hz) corresponds with spiking activity while low  $\gamma$  (<80 Hz) corresponds with localized network synchronization, including in those engaged in early sensory processing<sup>70</sup>. In simple visual tasks  $\gamma$  activity also superficially resembles  $\alpha$  activity, and is power modulated in correspondence with visual properties such as spatial frequency<sup>71</sup>. This stimulus-dependent modulation of  $\gamma$  is either reduced<sup>62</sup> or absent<sup>72</sup> in ASD, despite typical behavioral responses. In both cases this finding was interpreted to indicate a disturbed balance of excitation and inhibition (E/I) in the cortical networks recruited for early visual processing<sup>72</sup>. Gamma disturbances are not restricted to simple stimuli, however, and the  $\gamma$  response to complex<sup>73</sup> and illusory<sup>74, 75</sup> visual stimuli are similarly attenuated in ASD. Abnormalities in  $\gamma$  response to these perceptually complex stimuli are also more durable, indicating that slower perceptually focused processing is affected. An example of this can be found when children with ASD are asked to make perceptual judgments regarding Mooney faces. Mooney faces are two-tone face images with greatly reduced information content. In these tasks, participants must rely more heavily on feature integration for formation of a face percept than localized information. When asked to determine whether Mooney faces and matched images are faces, children with ASD present significantly reduced  $\gamma$  power and phase coherence in early activity (<150 ms post

stimulus)<sup>75</sup>. Gamma power in later stages of processing linked to perception (>200 ms post stimulus) also differs in both power and localization<sup>75</sup>. This is consistent with early stimulus processing networks being less specialized in ASD and that subsequent perceptual processing is also affected. Importantly, behavioral impairments in terms of response accuracy and speed emerge in this task. Similar findings can be found in a study utilizing contour defined shapes known as Kanisza squares and a younger ASD cohort<sup>76</sup>. In this study, children with ASD between the ages of 3 and 7 demonstrated significantly reduced evoked  $\gamma$  power during moderate to late time periods (120-270 ms). Neural activity in this time frame is believed to represent perceptual processes related to contour integration<sup>76</sup>. Together, these studies indicate that relatively localized  $\gamma$  band network recruitment and synchronization in response to simple visual inputs is altered in ASD. Furthermore, behavioral impairment emerges when visual stimuli become more complex and perceptual integration is required. Dysfunction in the  $\gamma$  band thus appears to correspond with deficits in visual perception tasks.

The pattern of reduced  $\gamma$  synchronization is not restricted to visual processing, and auditory investigations have demonstrated a nearly identical pattern of dysfunction. In discrimination tasks using pure tones, children with ASD have a reduced level of evoked  $\gamma$  power compared to TD children<sup>77-79</sup>. Importantly, this occurs despite evidence that spectral processing is intact in ASD<sup>22</sup>. Like the finding of attenuated  $\gamma$  power in response to spatial gratings, this indicates reduced special-

ization in the networks recruited during processing. This reduced  $\gamma$  power has also been found in a valproic acid rodent model, further establishing links between the ASD phenotype and altered neural synchrony<sup>77</sup>. Evidence of  $\gamma$  disruption can also be found in auditory entrainment paradigms. Typically,  $\gamma$  activity can be strongly entrained in auditory cortex using amplitude modulated sound<sup>80</sup>, and this effect is strongest near 40 Hz<sup>81</sup>. This is similar to the way repetitive visual stimulation can be used to entrain  $\alpha$  oscillations in visual cortex. Children and adolescents with ASD demonstrate a reduced amount of phase locking in the neural response to these amplitude modulated sounds<sup>82</sup> (**Fig. 2B**). Reductions in this auditory entrainment response have also been demonstrated in first degree relatives of individuals with ASD, implicating it as a phenotypic marker<sup>83</sup>. Altered synchronization in response to tones and auditory entrainment indicates that basic auditory sensory processing is altered in ASD, despite intact behavioral performance. In addition to this, recent evidence suggests that atypical auditory  $\gamma$  band function may contribute to behavioral deficits when perceptual demands increase. The level of  $\gamma$  power in the superior temporal gyrus, a core auditory area, was recently shown to be chronically elevated in a large ASD sample<sup>78</sup>. Furthermore, the level

of chronic  $\gamma$  power elevation corresponded with measures of language function in this study. This indicates that in addition to altered recruitment in response to auditory stimulation there is also an inability to suppress  $\gamma$  oscillations appropriately.

The ubiquity of  $\gamma$  oscillator dysfunction indicates that disturbance of high frequency synchronization is a robust feature of sensory processing in ASD. This body of research demonstrates that, for even the most basic sensory stimuli, recruitment of localized processing networks is different in ASD. For these simple stimuli, however, behavioral deficits are not obviously present. Visual illusions or speech comprehension tasks present stimuli with more complex features than tones or gratings, and these stimuli require increasing levels of perceptual integration to support task demands. Under these circumstances, the relationship between  $\gamma$  dysfunction and behavioral impairment in ASD becomes more apparent. An important parallel can also be drawn between these findings and studies of  $\alpha$  recruitment and suppression. It appears that network synchronization is less flexible in individuals with ASD, and that inability to appropriately recruit and suppress networks contributes to behavioral impairment.

## Hierarchical and Multi-Band Oscillatory Disruptions

Frequency bands other than  $\alpha$  and  $\gamma$  have been less interrogated as standalone contributors to perceptual disruption in ASD. Instead, they have been interrogated in investigations of oscillatory hierarchies and multi-band disruption across numerous frequencies. One critically important oscillatory hierarchy is based on the entrainment of  $\theta$  that encodes the slow amplitude modulations found in speech signals<sup>84, 85</sup>. Through oscillatory coupling,  $\gamma$  activity critical to speech perception depends on these slow  $\theta$  modulations<sup>86</sup>. Individuals with ASD are significantly less able to entrain to these slow speech amplitude modulations and lack this  $\gamma$  regulation<sup>87</sup> (**Fig. 2C**). Disruption of this entrainment and hierarchical coupling presumably results in degraded speech percepts leading to reductions in speech intelligibility, although this

correspondence needs to be more carefully tested in ASD. Additional evidence of similar hierarchical disruption in sensory processing in ASD is sparse, but the failure of  $\alpha$  activity to organize  $\gamma$  activity during visual processing has been demonstrated<sup>88</sup>. Additional work examining the integrity of oscillatory hierarchies in ASD is clearly needed. Disruption of  $\delta$  and  $\theta$  in sensory tasks without coupled  $\gamma$  dysfunction is even more limited; one example is that Isler and colleagues found a reduction in  $\delta$  and  $\theta$  connectivity between hemispheres in ASD in response to visual stimulation<sup>89</sup>. Failure of inter-hemispheric synchronization could contribute to failure of perceptual integration involving diffuse sensory evidence. Similar inter-hemispheric synchronization differences have also been found in the  $\beta$  band in response to repetitive visual stimu-

lation<sup>90</sup>. Unfortunately, these studies did not establish the perceptual or behavioral relevance of these connectivity differences. Further research on sensory driven inter-hemispheric connectivity differences is needed to determine if and how they correspond with sensory and perceptual phenotypes.

In addition to relatively underexplored hierarchical coupling deficits there is also limited evidence that multi-band oscillatory dysfunction may characterize sensory processes in ASD. Edgar and colleagues found that activity in the superior temporal gyrus across a large frequency range (4-80 Hz) was characterized by increased pre-stimulus power<sup>78</sup>. They suggest that this increased power characterizes either an inability to maintain 'neural tone', associated with reduced inhibition, or represents an inability to reset oscillatory state after a stimulus<sup>78</sup>. Both of these would result in a reduced signal to noise ratio by generating inconsistencies in stimulus locked activity. Supporting this, excessive broadband power was found to correspond with early  $\gamma$  abnormalities and later low frequency abnormalities in response to auditory stimuli. Importantly, this study had a very large sample size of

105 ASD children, supporting the reliability of the results. Further research is needed to determine the relevance of multi-band power elevations, correspondence with evoked responses, and the broader relationship to behavior. The finding of increased power over a wide range of frequencies in ASD also raises an important methodological issue; if intrinsic broadband power is chronically elevated, then procedures that determine power changes using a baseline systematically underestimate both evoked and induced power. Resting state investigations of ASD connectivity using EEG and MEG have frequently ignored this issue and often fail to report whether power measures are relative or absolute<sup>91</sup>. This has resulted in significant inconsistency in the results of these studies and fostered interpretational difficulties. More robust analysis approaches are needed to clarify the apparent link between resting oscillations, the autism phenotype<sup>92</sup>, and autism traits in the general population<sup>93</sup>. Improvements in analytical techniques<sup>94</sup>, as well as methods of moving connectivity analyses away from sensor based measures<sup>8</sup> may provide clarity on the nature of excessive broadband power and resting oscillations in ASD.

## The Neurobiology of Altered Oscillator Function in ASD

The consistent finding of impaired power modulation and reduced phase synchronization implies a common source rooted in the neurobiology of ASD. A leading proposal for this disruption is a combination of decreased inhibition and excessive excitation. The resulting imbalance of E/I is believed to lead to hyper-excitable and unstable neural networks<sup>95</sup>. Indirect evidence of decreased inhibition in ASD includes the high prevalence of comorbid epilepsy<sup>96</sup> and the recognition that many genetic risk factors for the disorder are related to inhibitory interneuron function<sup>97</sup>. More direct evidence of inhibitory dysfunction in ASD is present in the form of altered GABA receptor expression<sup>98</sup>. Depressed GABA expression has also been specifically noted in sensory processing regions such as the auditory cortex<sup>99, 100</sup>. The finding of denser and less organized mini-columns in ASD implies that structural organization may also contribute to inhibitory dysfunction<sup>101, 102</sup>. These studies indicate that

a number of functional alterations appear to contribute to an overall reduction of inhibition in ASD. Evidence of increased blood and cortical glutamate level in ASD indicates that excessive excitation may also contribute to imbalanced E/I<sup>103-105</sup>. It is important to note, however, that the role of excess excitation is still under debate, and both hyper- and hypo-glutamatergic rodent models display ASD like phenotypes<sup>106</sup>. How the glutamatergic system contributes to the disrupted E/I balance observed in rodent models<sup>107, 108</sup> is still under investigation.

Alterations in inhibitory function in particular align well with electrophysiological findings of oscillatory disruption. Gamma oscillations are known to arise from interneuron feedback, particularly in fast-spiking interneurons expressing parvalbumin<sup>109</sup>. Furthermore, much of the suggested function of  $\gamma$  rhythms depends on maintaining extremely high temporal precision in the activity

of these interneurons<sup>110</sup>. Disruption of the E/I balance thus logically results in a reduction of  $\gamma$  power and phase locking similar to that observed in many sensory processing tasks. It is still unknown how imbalanced E/I may contribute to inability to suppress  $\gamma$  oscillations or slower oscillatory mechanisms. Further, the overall relationship between rhythmic activity, neurotransmitter abundance, and cognitive processes is still an area of ongoing investigation. However, cortical GABA concentration is known to relate to the frequency of  $\gamma$  activity in response to visual stimulation<sup>111</sup>. Whether

the frequency of  $\gamma$  activity in response to sensory stimulation is reduced in ASD is currently unexplored, but strongly suggested by the recently discovered correspondence between peak  $\gamma$ , visual discrimination thresholds, and autistic traits<sup>112</sup>. This correspondence could potentially bridge known GABAergic biomarkers and sensory impairment in ASD. Overall, the concept of a generalized imbalance between excitation and inhibition in ASD has widespread empirical support. Significant work is needed to determine exactly how this imbalance contributes with perceptual function.

## Clinical Implications of Oscillator Dysfunction

Perturbations in sensory and perceptual function have been increasingly recognized as core features of ASD that contribute to lifelong disability<sup>1</sup>. The recognition of synchronization's role in pathology raises two critical questions: whether oscillations can be utilized for treatment evaluation, and whether oscillatory function itself is a potential avenue of treatment. There is currently a bustling industry of sensory interventions for ASD, but evidence for the effectiveness of these regimes ranges from nonexistent<sup>113</sup> to limited<sup>114</sup>. Furthermore, effectiveness is assessed using clinical methods, which are vulnerable to experimenter bias, frequently rely on parent report, and often lack a mechanistic basis. Prospective evaluative models should draw from examples in other fields, such as studies demonstrating that audiovisual training corrects deficits in early auditory  $\gamma$  power in children with language-learning impairment<sup>115</sup>. A similar approach is warranted in ASD to directly address whether sensory interventions improve phase locking or power modulation. Such an approach also promises to differentiate improvement due to the development of compensatory strategies from true remediation of neural dysfunction. Significant fundamental research is needed to support the development of utilizing oscillations in this way.

More speculatively, correcting oscillatory dysfunction in ASD may be a potential treatment target. There has been limited preliminary work on modifying oscillations in ASD in this manner. One

approach to doing so is attempting to correct E/I balance with repetitive transcranial magnetic stimulation (rTMS). Slow rTMS (<1 Hz) has been shown to reduce the level of excitability in stimulated cortex<sup>116</sup>, while fast rTMS (>1 Hz) increases the level of excitability<sup>117</sup>. Slow (0.5 Hz) dorsolateral prefrontal cortex rTMS was utilized by Sokhadze and colleagues to alter E/I balance in ASD<sup>118</sup>. Changes resulting from treatment were assessed with measures of oscillatory activity during processing of Kanisza squares. After six rTMS sessions over two weeks adolescents and adults with ASD showed significant normalization of evoked responses and induced  $\gamma$  activity<sup>118</sup>. This study indicates that correction of E/I balance and corresponding processing may be possible. The lack of behavioral correction in reaction times or accuracy, however, casts doubt on the efficacy of treatment. Utilization of rTMS in a clinical population also carries significant drawbacks. More intriguingly, modification of  $\gamma$  synchronization through brain machine interfaces has been demonstrated to impact perceptual processes<sup>119</sup>. Similar training has also been shown to impact other aspects of neural response such as spike timing in primates<sup>120</sup>. These studies raise the exciting possibility of using bio-feedback or perceptual learning paradigms to correct oscillatory disruption and perceptual deficits in ASD. Future research addressing whether correction of oscillations is effective for remediation of sensory function in ASD is clearly needed. Furthermore, significant methodological rigor should

be applied in such designs to link robust changes in behavior with neural plasticity. Well-construct-

ed research that meets these challenges may yield significant insight into new avenues of treatment.

## Conclusions and Future Directions

Disruption in oscillatory synchronization during sensory and perceptual processing is ubiquitous in ASD. These disruptions have been found in multiple sensory modalities and localized to disparate sensory processing regions. Furthermore, disruption occurs at frequency ranges associated with both long range ( $\delta$ ,  $\theta$ ,  $\alpha$ ,  $\beta$ ) and short range ( $\beta$ ,  $\gamma$ ) connectivity. For tasks that are not reliant on perceptual integration, reductions in synchronization are either irrelevant or beneficial in terms of discrimination thresholds, reaction times, and accuracy. For tasks requiring integration of disparate sensory information, reductions in synchronization prove more problematic. For these tasks, deficits in synchronization manifest as impairments in reaction time and accuracy. Based on this relationship, dysfunction of oscillatory synchronization should be considered as a biomarker of disruption in sensory processes and the bridge between altered biology and altered perception. The importance of these perceptual alterations cannot be overstated, and they have been hypothesized to contribute to the development of higher order social and cognitive deficits<sup>121</sup>. Processes dependent on merging sensory evidence such as multisensory speech perception may be particularly vulnerable<sup>30, 122</sup>. Given the significant ecological importance of such signals, further investigation of these processes is needed. The success of linking coordination between the  $\delta$  and  $\gamma$  bands to severity of verbal impairment and autism symptoms reinforces the power of such approaches<sup>87</sup>. Similarly, oscil-

latory coupling between  $\alpha$  and  $\gamma$  activity has been demonstrated to have high diagnostic value<sup>88</sup>. Extension of this research strategy to other paradigms is needed to firmly address if hierarchical coupling of oscillations is a globally disrupted mechanism in ASD. Failures in oscillatory coupling also constitute a reasonable but under-investigated explanation for intra-participant neural response variability that has been noted in both EEG<sup>63</sup> and fMRI<sup>123</sup> studies. Further research is needed to determine whether oscillatory coupling plays a role in this phenomenon. Additionally, causal relationships between oscillations and perception in ASD can now be probed with the advent of techniques such as transcranial alternating current stimulation<sup>124</sup>. Investigators have already used this technique to demonstrate how individual oscillatory frequency contributes to perception<sup>125</sup>. Causal relationships should be clarified through direct manipulation of oscillations intended to recreate the ASD perceptual pattern. Investigators pursuing such research should account for baseline power differences and report both relative and absolute power changes. Similarly, baseline phase locking and peak frequencies are currently underreported in the literature, providing interpretational difficulties. Despite these challenges, future research on oscillatory function in ASD may help clarify the etiology of perceptual deficits and their contribution to disability. It also holds significant promise for the development of new empirically validated treatments.

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# Developmental Dyscalculia: Competing Evidence for Underlying Neural Deficits

*Erik D. Wilkey*

## Abstract

Early math skills are a strong predictor of an individual's academic achievement, college entry, employment, and physical and mental health. Math skills can be impaired by a range of factors including poor education, low motivation, familial environment, and reading ability. However, a significant number of people with low math competence have the specific math learning disability Developmental Dyscalculia (DD). These individuals display difficulties with fundamental aspects of numerical processing from very early ages and continue to struggle with math. Despite the individual and societal costs of impaired math skills, little is known about the etiology of DD, and consequently, effective diagnostic and intervention tools remain elusive. Cognitive neuroscience offers a promising framework for disentangling the neural mechanisms underlying specific deficits in acquiring math skills. Many advances have been made in recent years in understanding how the typically developing brain processes numerical information that may begin to shed light on the neurological profile of people with.. For example, it is now well documented that the human brain contains topographic maps in the association cortex that represent numerical information in a similar fashion to the primary senses. The question the becomes—Is this system a principal source of difference between typically developing (TD) and DD individuals? Two main hypotheses offer mechanistic accounts of core neurocognitive deficits that could account for the DD profile. The current review will survey extant neuroimaging literature on DD and detail two competing theories of its underlying neurocognitive deficits.

**Keywords:** *numerical cognition, developmental dyscalculia, math learning disability, educational neuroscience, developmental cognitive neuroscience*

## Introduction

Numerical information is pervasive in modern life. From simple tasks, such as telling the time or giving the correct change back to a customer, to more complex demands, like long-term financial planning, individuals are tasked with an ever-increasing demand for mathematical fluency. Math skills measured at an early age are a strong predictor of an individual's academic achievement, college entry, employment, and physical and mental health<sup>1-3</sup>. On a societal level, small increases in a nation's numeracy rate are related to observable increases in GDP<sup>4</sup>. In the United States, over 1 in 4 adults is functionally innumerate, scoring below PISA's baseline for math skills that will enable them to participate effectively and productively in life<sup>5,6</sup>. The development of math competence can be impaired by a range of factors including

poor education, low motivation, familial environment, and reading ability. However, a significant number of people with low math competence have the specific math learning disability Developmental Dyscalculia (DD). These individuals display difficulties with fundamental aspects of numerical processing from very early ages.

Despite the individual and societal costs of impaired math skills, little is known about the etiology of DD, and consequently, effective diagnostic and intervention tools remain elusive. Compared to other developmental disorders, DD is severely understudied. For comparison, between 1985 and 2009, more than 16 times as many research papers were published on dyslexia<sup>7</sup> despite similar prevalence rates and severity indices. Since 2000,

the NIH has allocated \$107.2 million in funding for dyslexia research, but only \$2.3 million for DD. In order to develop a thorough understanding of the disorder, more research on the underlying neurocognitive profile of DD is necessary. The etiology of this profile may then be the topic of fu-

ture research that will aid in the development of enhanced diagnosis and intervention. The current review will survey extant literature on DD, detail competing theories of its underlying neurocognitive deficits, and suggest guiding principles to bring clarity to research on DD moving forward.

## Defining Developmental Dyscalculia

The Diagnostic and Statistical Manual of Mental Disorders (DSM) and the International Classification of Diseases-10 (ICD-10) categorize DD as a neurodevelopmental disorder with a biological origin (i.e. an interaction of genetic, epigenetic, and environmental factors) that manifests at the cognitive level as poor mathematical ability. DD is characterized by difficulties with processing numerical information, learning arithmetic facts, and performing accurate or fluent calculations<sup>8</sup>. As a developmental disorder, DD can typically be identified at the onset of formal mathematical instruction during the early school years. For recommended diagnosis, math ability should be persistently and substantially less than expected, given an individual's chronological age, intelligence, and education<sup>8-10</sup>. Math ability 1.5 standard deviations below average (<7th percentile), despite an IQ in the normal range, is frequently used as a diagnostic criterion. Using this criterion, it is estimated that 3-6% of the general population suffers from DD<sup>11,12</sup>. Recently, efforts have been made to move from a discrepancy model (lower math ability than that predicted by IQ) to a response to intervention model (continued low math performance in spite of remediation efforts)<sup>13</sup>.

Despite a working clinical definition born of necessity, the greater research community has not established a functional definition of DD. Depending on the publication, terms such as "Math Learning Disability", "Arithmetic Disability", and "Dyscalculia" are, at times used to contrast math disorders of differing severity, and at other times used interchangeably as synonyms. The lack of

consensus among researchers has resulted in a confusing array of DD samples. Table 1 details the selection criteria for all neuroimaging studies of DD to date listed in order of cutoff thresholds of standardized math tests, below which a sample is defined as DD. Studies primarily utilize samples with an IQ in the normal range, but math performance thresholds range from the 7th to the 37th percentile and are often not reported. Given that prevalence rates are estimated to be, at most, 6% of the general population, these liberal thresholds call into question the nature of the samples comprised in DD research. Future studies should be careful to adhere to a selection criteria characteristic of the persistent and severe impairment associated with DD.

Further, in order for diagnosis to lead to remediation and intervention, the DD label should shed some light on the causal origin and underlying neurocognitive mechanisms of the impairment. Therefore, the research community must approach DD in a manner that is theoretically and cognitively constrained. Cognitive neuroscience offers a promising framework for disentangling the underlying mechanisms of endogenous deficits for acquiring math skills. A small but growing body of neurocognitive and neuroimaging research suggests that atypical structural integrity, functional connectivity, and task-based recruitment of posterior parietal regions are associated with DD. Currently, three hypotheses offer preliminary explanations for mechanistic accounts of core deficits in processing numerical information that could account for the DD profile.

Author (year)	n= DD (TD)	DD math percentile rank	Age of DD (range or mean)	Imaging Method	Task Type
Kucian, K. (2006)	18 (20)	unreported	9.2 to 12.0	fMRI	approximate and exact calculation; magnitude comparison
Soltész, F. (2007)	7 (14)	unreported	mean = 17.4 (sd = 1.4)	EEG	static symbolic number comparison (reference to 5)
Rotzer, S. (2008)	12 (12)	unreported	mean = 9.3 (sd = 0.2)	sMRI	NA
Mussolin, C. (2010a)	15 (15)	unreported	mean = 10.5 (sd = 1.6)	fMRI	symbolic number comparison
Kucian, K. (2011)	15 (15)	unreported	3 <sup>rd</sup> & 6 <sup>th</sup> grade mean = 11.3	fMRI	nonsymbolic number comparison
Ranpura, A. (2013)	11 (11)	mean = 27 <sup>th</sup>	8 to 14	sMRI	NA
Cappelletti, M. (2014)	11 (22)	mean = 17 <sup>th</sup>	25 to 70	sMRI / fMRI	numerical judgment
Berteletti, I. (2014)	20 (20)	< 37 <sup>th</sup>	9 to 13	fMRI	multiplication; small/large
Rykhlevskaia, E. (2009)	23 (24)	< 37 <sup>th</sup>	7 to 9	sMRI	NA
Rosenberg-Lee, M. (2015)	16 (20)	< 25 <sup>th</sup>	7 to 9	fMRI	addition subtraction; number identification
Davis, N. (2009)	37 (27)	< 25 <sup>th</sup>	8 to 9	fMRI	exact and approximate addition
Ashkenazi, S. (2012)	17 (17)	< 25 <sup>th</sup>	7 to 9	fMR	addition; small/large
Han, Z. (2008)	20 (20)	< 20 <sup>th</sup>	mean = 10.8 (sd = 0.4)	sMRI	NA
Iuculano, T. (2015)	15 (15)	< 16 <sup>th</sup>	mean = 8.65 (sd = 0.47)	fMRI	addition (arithmetic verification)
Kaufmann, L. (2009)	9 (9)	< 16 <sup>th</sup>	mean = 9.6 (sd = 1.1)	fMRI	nonsymbolic number comparison
Kaufmann, L. (2009)	6 (6)	< 16 <sup>th</sup>	mean = 10.5 (sd = 2)	fMRI	numeric and non-numeric ordinality
De Smedt (2011)	8 (10)	< 16 <sup>th</sup>	mean = 11.65 (sd = 0.7)	fMRI	addition and subtraction
Dinkel, P. J. (2013)	16 (16)	< 10 <sup>th</sup>	6.5 – 10.5	fMR	nonsymbolic comparison; nonsymbolic exact calculation
Kovas, Y. (2009)	13 (13)	< 7 <sup>th</sup>	mean = 10.0	fMRI	nonsymbolic number comparison
Price, G. R. (2007)	8 (8)	< 7 <sup>th</sup>	mean = 11.4 (sd = 0.59)	fMRI	nonsymbolic number comparison
Rotzer, S. (2009)	10 (11)	< 7 <sup>th</sup>	mean = 10.4 (sd = 1.2)	fMRI	spatial working memory (Corsi block tapping task)
Kucian, K. (2013)	15 (15)	< 7 <sup>th</sup>	mean = 10.0 (sd = 1.2)	sMRI	NA

\*\* Indicates studies that utilize percentile cutoffs in coherence with DD prevalence rates.

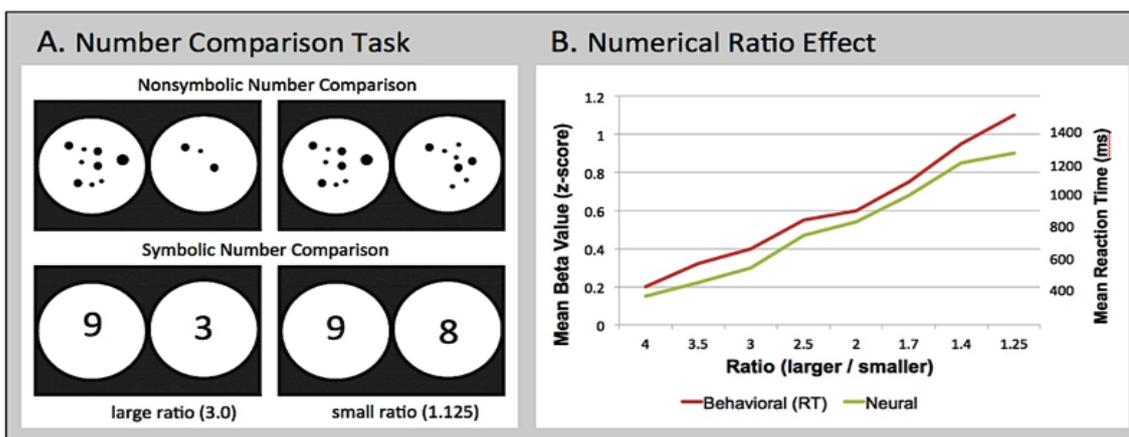
## Magnitude Representation Deficit Hypothesis (MRD)

Perception of numerosity has been documented in animal species ranging from fish<sup>30</sup> and birds<sup>31</sup> to nonhuman primates<sup>32-34</sup>, indicating that numerical coding is likely an inherent property of the human brain<sup>35</sup>. Infants as young as 6 months old are able to discriminate sets of objects on the basis of numerosity<sup>36,37</sup>. Electrophysiological recording in nonhuman primates have identified populations of neurons in the lateral prefrontal cortex and ventral intraparietal sulcus (IPS) that code for numerosity irrespective of the sensory modality of stimuli (i.e. auditory or visual)<sup>38-40</sup>. Human fMRI studies subtracting numerosity-dependent neural activity from visual control tasks frequently isolate activity in the bilateral IPS as well<sup>41,42</sup>. The MRD hypothesis proposes that DD is caused by a domain-specific impairment of the capacity to represent and manipulate numerical information<sup>43-45</sup>. Further, many researchers suggest that deficits in symbolic number representation, arithmetic fluency, and higher order mathematical thinking emerge from a core deficit in this system<sup>14</sup>.

### *Numerical magnitude processing in the typically developing brain*

In 1967 Moyer and Landauer observed that when comparing two digits side-by-side, reaction time and error rates decrease as the numerical distance between stimuli increases.<sup>46</sup> For example, people more quickly and accurately identify 9 to be greater than 4 than they do 5 to be greater than 4. This so-called “distance effect” has been replicated in hundreds of studies across multiple formats of

number representation<sup>47</sup> including nonsymbolic numerical stimuli (see **figure 1A** for an example of a symbolic and nonsymbolic comparison task). Both distance (e.g.  $9 - 4 = 5$ ) and ratio (e.g.  $9/4 = 2.25$ ) between numerical stimuli have been used somewhat interchangeably in the literature. However, the relative magnitude of number sets does impact perception of numerosity and the data exhibit a tighter parametric relationship with ratio. Larger ratios are similar to larger distances in that they exhibit quicker and more accurate responses than smaller ratios/distances. To avoid confusion, this review will refer to the “ratio effect” and “ratio” as a property of the stimuli regardless of specific metrics used in individual studies. The ratio effect is found not only at the behavioral level, but can also be measured in neural activity as imaged by blood-oxygen-level dependent (BOLD) signals through fMRI. It is referred to as the neural ratio effect. Brain regions such as the IPS (see **figure 2**) show greater activation during the discrimination of numerical stimuli differing by smaller ratios than greater ratios<sup>48,49</sup>. Further, because behavioral performance is thought to represent the acuity of the underlying neural representations<sup>50-52</sup>, researchers have investigated their psychometric properties as an index of individual variability in number processing foundational to mathematical ability<sup>53</sup>. Large scale behavioral studies find significant correlations between math achievement and individual differences in both symbolic and nonsymbolic number



**Figure 1.** A) Example of stimuli from number comparison task. B) Hypothetical data demonstrating a typical behavioral and neural ratio effect. Reaction time, accuracy, and beta values for BOLD signals increase as the ratio of the two numerical stimuli compared decreases.

discrimination<sup>54–57</sup>. Similarly, greater neural ratio effects in the left IPS during symbolic magnitude comparison positively correlate with arithmetic performance<sup>58</sup>.

However, in a formalized system, there are more components to numerical processing than simply the underlying semantic content. The most prominent model associating different aspects of numerical cognition with specific brain circuitry remains the ‘Triple Code Model’ proposed by Stanislas Dehaene in 1995<sup>59</sup>. The Triple Code Model hypothesizes that there are three different representational systems recruited for numerical tasks. The quantity code is associated with processing in the bilateral IPS and codes for nonverbal semantic representation of numerical magnitude. The visual number code is involved in visually encoding strings of numbers. This system is thought to involve brain regions belonging to the ventral visual stream, including the bilateral occipito-temporal cortex. The verbal number code is associated with processing within the left frontal and temporal language areas. In this system, numerals are represented lexically, phonologically, and syntactically. This triple code model was developed using dissociable deficits in numerical processing found in patients with brain lesions. For example, a patient with lesions in the left inferior frontal and superior/middle temporal gyri demonstrated specific loss of the ability to add numbers verbally while maintaining the ability to approximate quantities and identify strings of digits<sup>59</sup>.

Further complicating the landscape of number representation in the brain is that mathematics is a set of skills learned through schooling over the course of development. Though the most dramatic functional organization of brain networks happens during early childhood<sup>60,61</sup>, it is believed that the human brain is not fully mature until an individual’s late twenties<sup>62</sup>. Some researchers suggest that the IPS is a higher-order visual processing center dedicated to domain general magnitude differences, including properties such as size<sup>63,64</sup>, luminance<sup>65,66</sup>, and ordinality<sup>67–69</sup>. In this model, the IPS becomes specialized for numerosity discrimination through formal training. However, evidence for a developmental shift of IPS

processing remains mixed. One study comparing 4-year-old children to adults showed no group differences in IPS activation due to processing of numerical information vs. non-numeric information<sup>70</sup>. Still, regional increases in BOLD signal based on contrasts between numeric and non-numeric stimuli tell a limited story. Subsequent studies focused their analysis on activation in the IPS modulated by stimuli of differing numerical ratios. Using both symbolic and nonsymbolic number comparison tasks, Ansari & Dhital (2006) found that elementary school children demonstrated a greater neural ratio effect in prefrontal regions, compared to adults. Adults demonstrated a greater neural ratio effect in the IPS<sup>48,71</sup>. Similar shifts from frontal to parietal networks through development have also been documented during mental addition and subtraction<sup>72</sup>. Both studies have been interpreted as indicating that the specialized role of the IPS for processing numerical information increases over the course of development.

#### *Numerical magnitude processing in the atypically developing brain*

Historically, the earliest evidence for functional specialization of posterior parietal regions for numerosity coding comes from clinical case studies. Patients with lesions to the left or right parietal lobe exhibit specific calculation deficits for approximation, addition, and subtraction<sup>73,74</sup>. Often, these deficits are dissociable from memorized arithmetic factual knowledge such as access to multiplication tables<sup>75,76</sup>. In support of the MRD hypothesis, DD children share several characteristics with clinical patients that have acute parietal lesions and acquired dyscalculia. Compared to typically developing (TD) children, DD children show poorer number acuity<sup>52,77,78</sup>, decreased structural integrity of the bilateral IPS and associated white matter tracts<sup>16,20</sup>, and share behavioral deficits superficially unrelated to mathematics such as finger agnosia and problems with left-right discrimination<sup>44,79</sup>.

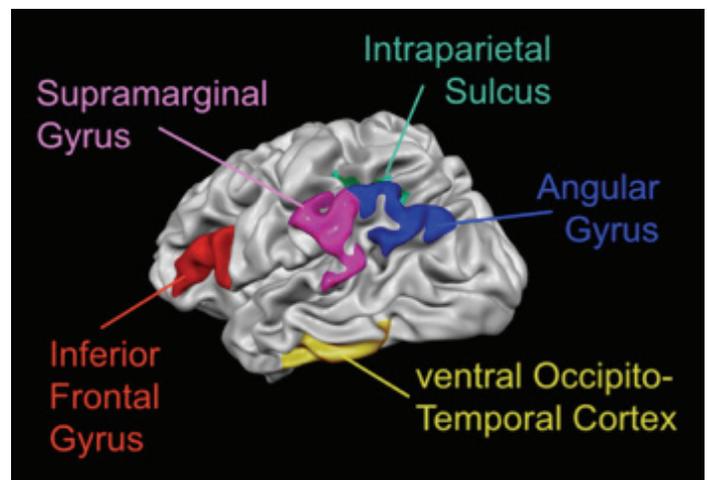
Only 17 studies to date have investigated functional or structural brain differences in children with pure DD (without a comorbid disorder such as ADHD or dyslexia). As previously mentioned, they range widely in selection criteria (**Table 1**). Though the sample heterogeneity makes it difficult

to derive a consensus view, many lend support to the MRD hypothesis. Most tasks within these studies may be broken down into two broader categories. Number comparison tasks are two-alternative forced choice tasks where participants indicate the greater value of simultaneously or consecutively presented arabic digits (symbolic) or groups of objects (nonsymbolic). Simple arithmetic tasks are two-alternative forced choice tasks whereby participants indicate the correct solution to an addition, subtraction, or multiplication problem.

Studies utilizing nonsymbolic number comparison tasks generally report greater levels of activation in multiple brain regions in DD groups. Specifically, both Kaufmann et al.<sup>24</sup> and Dinkel et al.<sup>26</sup> found higher activation in posterior parietal areas for children with DD compared to controls. However, Kucian et al., found no group differences<sup>14</sup>. When analyzing group differences in neural ratio effect, rather than analyzing activation differences in a numerical comparison task vs. control task, the findings are more cohesive. Two fMRI studies, utilizing nonsymbolic and symbolic comparison tasks respectively, found that numerical ratio modulated activity in the right IPS in TD but not DD children<sup>28,80</sup>. One further study reported a neural ratio effect in posterior parietal regions for both TD and DD children<sup>18</sup>, but DD children differed by increased neural ratio effects in the right fusiform gyrus and bilateral supplementary motor area. Interestingly, Soltész et al. found evidence of an early event related potential (ERP) ratio effect (210-300ms) in both TD and DD children, but TD children alone demonstrated a later ERP ratio effect (400-440ms) focused generally in the right parietal lobe<sup>15</sup>. The authors interpreted these findings as evidence that early automatic processing of digits was similar in both groups, but that differences arose during more complex, controlled processing<sup>81</sup>. Comparing this group of results to Ansari et al.'s finding that the neural ratio effect was greater in the left IPS of TD adults than TD children, the overall trend of imaging studies indicates that the DD population has a developmental deficit in parietal systems specialized for numerical representation. Further corroborating the parietal deficit hypothesis, MRI guided trans-cranial magnetic stimulation applied to the right IPS (triple pulse repeated

TMS at 220ms, 320ms, and 420ms after stimulus presentation) was found to induce DD-like performance in automatic perception of numerosity<sup>82</sup>.

results mirror the trends found in number comparison studies indicating that DD children have stronger activation of posterior parietal regions during number related tasks, but reduced difficulty-based modulation<sup>14,83</sup>. Based on this evidence, it would seem that structural and functional abnormalities of the posterior parietal regions, most frequently the bilateral IPS, underlie a deficit in numerical magnitude processing which manifest as a deficit in more complex math skills like arithmetic.



**Figure 2.** Notable regions of the brain implicated in neuroimaging studies of numerical cognition and potential sources of atypical processing in DD presented on the left cerebral hemisphere.

### *Criticism of the Magnitude Representation Hypothesis*

The MRD hypothesis is subject to multiple criticisms. If DD is a deficit of one's ability to represent magnitude independent of format, then DD individuals should always exhibit impaired performance in the nonsymbolic and symbolic number comparison task alike. However, not all studies find a correlation between nonsymbolic number comparison deficits and DD status (for a review<sup>56</sup>). Further, when alternately run in a step-wise regression, performance on symbolic comparison tasks (not nonsymbolic comparison) accounts for most of the variance<sup>84</sup>. Secondly, neuroanatomical differences between DD and TD individuals are not limited

to the IPS, but rather extend to other parietal areas, frontal regions, and the supplementary motor area<sup>83</sup>. Many of these findings are consistent and unaccounted for by the MRD hypothesis. Thirdly, most of the functional literature applies reverse inference<sup>85</sup> relying on other studies for behavioral

correlations with no direct causal link being established. This is important because the IPS has been linked to visual processing of various kinds. Any study hoping to draw a direct correlation between IPS modulation and numerical processing must adequately control for alternative explanations.

## Symbol Access Deficit Hypothesis (SAD)

It has been suggested that over the course of development, children learn to link nonsymbolic magnitude representations with number words and arabic digits<sup>86-89</sup>. In other words, they learn to map the system for nonsymbolic representations with a higher precision symbolic system for representing numerical magnitudes. The SAD hypothesis suggests there is a breakdown in this mapping system rendering DD individuals with an intact core magnitude system less able to access high precision symbolic representations. This would account for the higher correlation between symbolic number comparison tasks and mathematics ability<sup>90</sup> and the findings of Rousselle and Noel (2007) that did not link DD with an impairment of the magnitude representation system<sup>84</sup>.

### *Symbolic representation of numbers in the typically developing brain*

Symbolic number comparison tasks are thought to tap into implicit associations between symbols and corresponding magnitude representation. In contrast, symbol mapping tasks attempt to explicitly measure numerical mapping ability by forcing the individual to transcode between formats<sup>88</sup>. During symbol mapping tasks, participants are presented with a target number in one format (i.e. an Arabic digit or nonsymbolic array) and then asked to choose the same number in the opposite format from two choices. One choice is an exact match to the target. The other is a distractor and is incorrect by a varying degree. Similar to number comparison tasks, symbol mapping efficiency is measured using accuracy and reaction time. Three studies have found a robust relationship between symbol mapping and math ability. One study in adults found that exact number precision<sup>91</sup>, as measured by a symbol mapping task and a symbolic comparison task, was enhanced in individuals with advanced mathematical train-

ing. In the same participants, performance on a nonsymbolic magnitude comparison task was not enhanced. The authors took this to indicate that formal training exclusively enhances exact, symbolic number representation without affecting basic magnitude representation. Mundy and Gilmore (2009) found that symbol mapping efficiency in TD children ages 6-8 correlated with math ability as measured by an untimed test of school math achievement over and above the variance accounted for by symbolic and nonsymbolic comparison efficiency<sup>88</sup>. A further study reported similar findings in a task incorporating lower numbers (1-9) with timed and untimed tests of arithmetic<sup>92</sup>. Together, these three symbol mapping studies demonstrate that individual variability in transcoding between symbolic and nonsymbolic number systems is linked to math performance.

There is also evidence to suggest that, once developed, nonsymbolic and symbolic systems may operate independently from one another. In a series of cross-format number transcoding tasks, Lyons, Ansari, and Beilock (2012) found that numerical symbols operate primarily as an associative system in which relations between numerical symbols eventually overshadow those between symbols and their quantity referents<sup>93</sup>. The Lyons study suggests that the symbolic system begins to operate independent of core magnitude representation in the natural course of development. If this is the natural course of development, it would be plausible that individuals with low math ability never dissociate these two systems, and maintain an inefficient link between the two systems. Alternatively, impaired symbolic number comparison performance might indicate a less efficient mapping of the symbolic number system onto the core magnitude representation system during its original development.

*Symbolic representation of number in the atypically developing brain*

To date, Mundy and Gilmore's symbol mapping task<sup>88</sup> has not been tested on a DD sample. However, behavioral findings from the symbol mapping literature fit nicely with heretofore unexplained (or under-explained) neuroimaging results. According to the MRD hypothesis, all deficits would result from an irregularity in the core magnitude system, located in the bilateral IPS. However, both the angular gyrus (AG) (see **figure 2**) and supra-marginal gyrus (SMG) are frequently implicated in studies of numerical processing<sup>94,95</sup>. Lesions of the AG are associated with symptoms of Gerstmann Syndrome, which include finger agnosia, the inability to read, the inability to use arithmetic operation, and left-right confusion<sup>96</sup>. In a study testing competency for solving single-digit and multi-digit multiplication problems, individuals with higher mathematical ability displayed stronger activation of the left AG while solving both types of problems<sup>97</sup>. The role of the AG in symbol processing was directly assessed by Price and Ansari (2011) by comparing activation in response to novel symbols and known symbols (digits and letters)<sup>98</sup>. The AG showed increased activation for known symbols compared to novel symbols. In the only study to date that successfully classified individuals according to DD diagnosis exclusively through task-related BOLD responses, the AG was identified as a marker of DD<sup>26</sup>. Based on the importance of the AG in language processing and its evident role in learned arithmetic facts, the AG seems to be tightly coupled with the IPS as a processor of symbolic number and likely also plays a role in symbol mapping. As a marked identifier of DD diagnosis, these studies could support the idea that atypical processing of the AG underlies a deficit in access to symbolic representation of number, and consequently a deficit in math ability.

*Criticism of the Symbol Access Deficit Hypothesis*

The symbol mapping hypothesis cannot account for the consistent correlation observed between math ability and acuity of format-independent magnitude representation<sup>55</sup>. Furthermore, it has gone unnoted thus far that the most consistently cited study linking DD to low performance in a symbolic comparison task in the absence of a non-symbolic impairment (Rousselle & Noel, 2007) used two symbolic number tasks as part of the selection criteria for defining the DD population. This choice effectively ensured that the group would exhibit an impairment on symbolic number tasks. It is unsurprising that participants defined as having DD on the basis of their performance on symbolic number tasks performed lower exclusively on a symbolic number comparison task. Further, if access to a symbolic representation of number is the underlying deficit for DD, how is this deficit domain-specific to mathematics? Symbolic representation of semantic information is not unique to math. For example, learning to read requires learning the alphabet, another symbol set. In most studies of DD individuals to date, individuals with other identified disorders such as ADHD, dyslexia, or other neurological condition are excluded from the participant pool in order to avoid confounds. However, it should be noted that nearly 40% of individuals with DD also have dyslexia<sup>99</sup>. Comorbidity between the two is likely an important facet of DD itself. This exclusion criteria leaves an important issue completely ignored. A mechanistic account for DD that involves symbolic representation of semantic content could begin to account for the high comorbidity in reading and mathematics disorders. This idea is highly speculative and the question of comorbidity as a result of domain general symbol access deficits must be explored empirically.

## Domain-General Hypotheses

Many researchers argue that domain-general cognitive deficits are the underlying cause of poor arithmetic performance in DD individuals. The disorder has been linked to deficits in phonological ability<sup>100</sup>, inhibitory control<sup>101-103</sup>, spatial processing<sup>104</sup>, verbal and visuospatial working

memory<sup>105,106</sup>, and attention<sup>107,108</sup>. Due to these correlations, some researchers propose that DD originates from one or more domain-general deficit. Working memory and spatial processing are two cognitive domains that frequently correlate with math ability and DD diagnosis. In a study of 12 stu-

dents in 3rd and 4th grade who performed poorly on a test of arithmetic and age-matched peers, researchers administered a battery of 10 working memory tasks<sup>109</sup>. Executive and spatial aspects of working memory were determined to be important indicators of poor arithmetic attainment. Neuroimaging experiments have also linked working memory deficits and DD. For example, Dumontheil and Klingberg<sup>110</sup> demonstrated that activity in the left IPS correlated with working memory capacity and predicted arithmetic achievement two years later.

To summarize, evidence from research on DD indicates it is a disorder with specific impairments of math ability, but DD status also frequently cor-

relates to domain general deficits. By its very definition, DD is a domain-specific disorder and the logic of searching for a domain-general mechanism seems misguided. It would, however, stand to reason that individuals who have difficulties with mathematical computations overcome those difficulties by developing compensatory strategies based on other cognitive faculties such as working memory or attention. If someone had a working memory or attention deficit in addition to DD, then that individual would no longer be able to compensate and poor performance in mathematics could result. This highlights the importance of determining universal criteria for defining DD samples.

## The Causes of Developmental Dyscalculia

Unfortunately, detailing the neurocognitive profile of DD does not immediately belie its developmental origin. The three hypotheses detailed in this review are mostly descriptive in nature, and very little is known about the underlying cause of the disorder. The preponderance of evidence is beginning to indicate that genetics and early developmental factors both contribute to increased risk for DD. One recent study found no link to premature childbirth in DD individuals, but did find an increased risk of DD if the child was classified as “small-for-gestational age”<sup>111</sup>. This might indicate that prenatal factors such as fetal growth restriction could be a developmental contributing factor

for DD. Elevated risk for DD when a family member has been diagnosed also indicates that genetics plays a role<sup>112</sup>. Based on twin studies, mathematical disabilities occur in monozygotic twins with a concordance of 70% and in dizygotic twins with a concordance of 50%<sup>113,114</sup>. Considering that autism is thought to be highly inheritable, with a monozygotic twin concordance rate of around 60%<sup>115</sup>, the link to genetics as a causal factor is likely. With an understanding of the dynamics of epigenetic phenomena, continued study of biological development and home/school environment might reveal more about the causes of DD.

## Conclusions and Future Directions

Individuals with DD exhibit clear deficits in processing numerical information, learning arithmetic facts, and performing accurate or fluent calculations with a severity that disrupts normal functioning in everyday activities. Despite the enormous individual and societal impact of the disorder, DD research remains comparatively understudied. For better and earlier diagnosis the underlying neurocognitive profile must be more clearly understood. Only with a thorough understanding of the nature of the disorder will interventions become increasingly effective. Though progress has been made, research efforts thus far have been encumbered theoretical and methodological

shortcomings including, most pervasively, the liberal cutoff thresholds for identifying DD samples. Because low math performance can be attributed to a variety of causes unrelated to DD, more rigor must be applied in assuring the severity and persistence of impairment of math ability in DD samples matches that characterized by the disorder.

Cognitive neuroscience has begun to offer hypotheses about the underlying nature of DD. At this point both the MRD and the SAD hypothesis offer mechanistic accounts of unique behavioral and neurocognitive attributes of DD populations. The MRD hypothesis proposes that DD is caused

by a domain-specific impairment of the capacity to represent and manipulate numerical information. Animal research and research on TD populations suggests that the bilateral IPS codes for numerical information. While structural and functional MRI as well as EEG data indicate atypical processing of the IPS in DD populations, the results are not exhausted by the MRD account. Further, the SAD hypothesis suggests there is a breakdown in the mapping system from core magnitude representations to symbolic number, rendering DD individuals with an intact core magnitude system less able to access high precision symbolic representations and, in turn, complete mathematical operations. This would explain the higher correlations of symbolic measurements of math ability with DD.

Moving forward, it seems appropriate that these systems for representing number should be investigated both independent of one another, but also as inter-dependent systems. Isolated deficits in different brain systems could lead to subtypes of the DD profile<sup>116,117</sup>. Furthermore, new techniques in network connectivity analyses have already provided insights in the interrelatedness of constituent systems for processing number<sup>118</sup> with other brain networks. More clearly understanding the interrelation of number processing systems with networks of attention, working memory, and cognitive control is likely to further provide insight into the complex nature of DD and the development of effective treatments.

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# GABAergic Regulation of Developmental Migration of Neocortical Interneurons

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## Abstract

Neurons populating the neocortex consist mainly of glutamatergic projection neurons and GABAergic interneurons. Though a minority to projection neurons, these interneurons are incredibly diverse and are essential to proper circuit function. Their development depends on an elaborate migration from subpallial regions into the neocortex. Multiple cues direct this migration and promote migratory behavior of interneuroblasts.  $\gamma$ -Aminobutyric acid (GABA) is particularly important in this regulation, providing either a migratory signal or a signal to exit migration, depending on the changing intracellular  $[Cl^-]$ . This review briefly discusses types of interneurons, then overviews mechanisms guiding their developmental migration, especially focusing on GABAergic signaling.

## Introduction

Multiple neurological and psychiatric diseases, including epilepsy, schizophrenia, and autism, originate from impaired network connections within the cerebral cortex. One significant cause of this defect is improper migration and circuit integration of interneurons arising during developmental or adult neurogenesis. Indeed, while interneurons comprise only about one-quarter of cortical neurons, their morphological, molecular, and physiological diversity imbues them with the ability to interconnect projection neurons and control a vast array of network functions. Their migration and maturation depend on both the cues encountered in the extracellular environment, many of which are present only during development, and the transcriptional program specific to the cell. Thus, elucidating the underlying cell extrinsic and intrinsic events is crucial for the understanding of these processes and pathophysiology of the associated disorders. The majority of interneuronal progenitors originate within the subpallium

and complete a long migration from the medial ganglionic eminence through the marginal and intermediate zones, and finally the cortical plate. GABA<sub>A</sub> receptor (GABA<sub>A</sub>R)-mediated activity of interneuron progenitors is critical for regulating their migration and maturation. Namely, decrease in intracellular  $[Cl^-]$  during interneuronal maturation marks the transition from migratory to sedentary behavior. Subsequently, GABAergic signaling continues to influence interneuronal maturation through chemotaxis in synaptic development. The different roles of GABAergic signaling are carried out by a temporally and spatially varying subunit composition of GABA<sub>A</sub>Rs. In this review, I provide a brief overview of neocortical interneurons, their developmental migration, and the known mechanisms regulating this migration. I particularly focus on the role of GABAergic signaling in providing initially a motogenic and subsequently a sedentary cue, and the hypothesis of changing interneuronal GABA<sub>A</sub>R expression in regulating these signals.

## Defining Interneurons

Neurons populating the mammalian cortex are mainly comprised of two general cell types – projection neurons and interneurons. These differ substantially by their morphology, major presynaptically-released neurotransmitter, and develop-

mental origin. Projection neurons are the principal cortical neurons. They are morphologically characterized by large, pyramidal-shaped soma, a single lengthy axon, and distinct apical and basal dendritic arborization, typically having just one

dendrite apically. Respective of their name, axonal processes of projection neurons travel distantly across cortical layers and into other brain regions, such as the thalamus, distant cortical regions, brainstem, and the spinal chord. The primary presynaptically released neurotransmitter of cortical projection neurons is glutamate, giving projection neurons excitatory signaling properties. Interneurons are a minority of cortical neurons. The primary morphological features of interneurons are a small soma, extensive dendritic arborization lacking apical/basal distinction of projection neurons, and a short axon. As such, interneurons specialize in locally interconnecting neuronal projections and regulating circuit activity. The majority of interneurons have few or no dendritic spines and presynaptically release the inhibitory neurotransmitter GABA, providing the main cortical inhibitory signal in juxtaposition to excitation of projection neurons. This cortical property is in contrast with inhibition in other brain regions, such as the nRT

of thalamus, the basal ganglia, and the cerebellum, that do contain GABAergic projection neurons. Interneurons comprise 10-30% of total cortical neurons, depending on species and area; for instance, in rats, 10-15% of cortical neurons are GABAergic, while in primates, 20-25% are (DeFelipe et al., 2002). They populate all of the cortical layers with relative homogeneity, but layers 2 and 5A have the highest density, correlate with low spike rates of excitatory neurons in these regions (Meyer et al., 2011). Distinct in local axonal arborization, interconnection, and inhibitory signaling, GABAergic interneurons are a specific class of cortical neurons. Thus, although other non-pyramidal neurons with local axonal arborization are observed within the cortex, such as the glutamatergic spiny non-pyramidal cells inhabiting mainly the middle layers, the term “interneuron” and the synonymous “local circuit neuron” refer specifically to GABAergic non-pyramidal cells (DeFelipe et al., 2013).

## Characterization of Interneurons

Interneurons possess a rich morphological, physiological, and molecular heterogeneity. The widely varying interneuronal properties dispose them to filling a numerous variety of specialty functions in circuit regulation (Wester and McBain, 2014), an essential role responsible for drawing attention to researching interneurons. To deal with this vast functional and phenotypic heterogeneity, elaborate classification has been developed initially by the Petilla group (PING et al., 2008), followed by a more modern classification (DeFelipe et al., 2013).

Morphologically, distinct types of interneurons have been distinguished based mainly on dendritic and axonal arborization, axonal targets, direction of projections, and cortical layer where the soma is found. The most recent interneuronal classification by DeFelipe et al. (2013) thus lists eight distinct types (refer to Table 1), acknowledging additional “common interneurons” that do not belong to a specific type, and the existence of additional, “other” potential types.

Physiologically, interneurons are characterized by their action potential-firing properties. Six

main categories exist based on spiking frequency of interneurons when a step of continuous depolarizing current is administered to the interneuron by an electrode: fast, regular non-adapting, regular adapting, regular accelerating, irregular, and intrinsically bursting. Within these categories, there are subcategories reflecting delayed, bursting, and stuttering spiking behaviors.

Cytochemically, cortical interneurons can be reliably identified by expression of GABA synthesizing enzymes glutamate decarboxylase 65 (GAD65) and GAD67. Almost all of the GAD67 positive cortical neurons are further subdivided by parvalbumin (PV), somatostatin (SST), or serotonin receptor (5HT3aR) non-overlapping expression (Lee et al., 2010). The distribution between the categories is fairly even - within the somatosensory cortex of the rat, Lee et al. (2010) found 40% of interneurons expressing PV, 30% SST, and 30% 5HT3aR. Additional sorting is possible based on neuropeptide Y (NPY), cholecystokinin (CCK), calcitonin (CR), vasoactive intestinal peptide (VIP), and calbindin (CB) expression. According to the classification of DeFelipe et al. (2013), five distinct groups appear: PV+,

SST+, NPY+ SST-, VIP+, CCK+ NPY- VIP-. According to Lee et al. (2010) and Rudy et al. (2010), the latter three categories would be within the 5HT3aR label.

Interneuron Type (DeFelipe et al., 2013)	Morphology (DeFelipe et al., 2013)			Typical Firing Properties	Developmental Origin	Main Cytochemical Marker Expression
	Soma Location (Layer)	Dendritic Properties	Axonal Properties			
Chandelier	II-VI	multipolar or bitufted	"Candlestick"-like short vertical rows of boutons, synapse on axonal initial segment of pyramidal cells	Fast-spiking	MGE	PV
Arcade	II-VI	multipolar or bitufted	Predominantly vertical arbors, long descending collaterals	Regular adapting	CGE	5HT3aR, VIP
"Cajal-Retzius"-like*	I	multipolar or bitufted	Long horizontal collaterals within layer I	uncharacterized	uncharacterized	uncharacterized
Large basket	II-VI	multipolar or bitufted	Horizontally oriented axon collaterals up to several hundred µm in length, numerous curved pre-terminal axon branches synapsing somata and proximal dendrites of targets; in addition, frequently have one or more descending axonal branches	Fast-spiking	MGE	PV
Neurogliaform	II-VI	multipolar	Very small and dense arborization proximal to the parent soma	Delayed regular non-adapting	CGE	5HT3aR
Common basket	II-VI	multipolar or bitufted	Numerous curved pre-terminal axon branches	varied	MGE	SST (varied regular-spiking) or PV (fast-spiking)
Horse-tail	II-III	Multipolar, bitufted, or bipolar	Bundled, tightly intertwined lengthy descending collaterals	Regular-spiking and burst-spiking	CGE	5HT3aR, VIP
Martinotti	II-VI, but V has the highest concentration	Multipolar, bitufted, or bipolar	Two ascending axonal arbors; first near the parent soma; second with a dense or diffuse plexus at a distance variable from being within the same layer to ascending as far as layer I	Regular adapting or intrinsically bursting	MGE	SST
Common type Other, not yet defined	I-VI	Multipolar, bitufted, or bipolar	Equal numbers of horizontal and vertical axonal projections	Varied	varied	Varied, but not PV

**Table 1.** Currently distinguished types of neocortical interneurons. Main morphological cell types are on the left as they appear in the most currently proposed nomenclature by Felipe et al., 2013. Developmental origin, cytochemical marker expression, and typical firing properties are on the right. As the classification of interneurons is still underway, distinction of a morphological type with specific cytochemical and electrophysiological properties is more a general rule rather than absolute grouping; additionally, some cells are defined based on all three properties. For more detailed information, view the cited reviews, especially Markham et al., 2004. Terminology: Multipolar - multiple extensively branched dendrites around the soma. Bitufted - two main dendrites running in opposite directions for a short distance before resolving into two dendritic tufts. Bipolar - two main dendrites running in opposite directions for a long distance with few dendritic collaterals before resolving into dendritic tufts. MGE - medial ganglionic eminence, CGE - caudal ganglionic eminence. \*Proper Cajal-Retzius cells are glutamatergic cells with long horizontal axonal collaterals that are found in layer I of developing cortex. GABAergic interneurons with similar morphology also exist and are not proper Cajal-Retzius cells, but are sometimes described as such morphologically. (Nomenclature according to DeFelipe et al., 2013; interneuronal properties reviewed by Markham et al., 2004; Rudy et al., 2010; Wonders and Anderson, 2006; Kepecs and Fishell, 2014)

Distinct morphological types of neocortical interneurons associate with specific cytochemical markers and physiological properties, though there is a high degree of overlap, and some cell types associate and classify better than others. Unfortunately, systematization of interneuronal types based on multiple properties across reports from different research groups is difficult due to inconsistency in morphological classification and physiological nomenclature, which is an issue that the aforementioned classification attempts aim to rectify. Additionally, high interneuronal complexity and unclear boundaries between cell types complicate classification attempts. Thus, an attempt to classify all interneuronal types is under way, but currently is incomplete. Markram et

al. (2004) and, most recently, Kepecs and Fishell (2014) provide the best current sorting, including overlap between all three of the categories, and address extensively all known interneuronal types.

In summary, it is well defined in the field that PV labels fast-spiking chandelier cells and basket cells, though their actual firing properties still differ, and not all basket cells are PV+. Martinotti cells are the main SST+ interneuron type, some of which express CR, and are either regular adapting or intrinsically bursting. 5HT3aR labels the most heterogeneous population of interneurons, including neurogliaform and horsetail cells (Rudy et al., 2011). **Table 1** outlines these general trends.

## Interneuronal Development

In humans, cortical GABAergic circuit development begins mid-gestation and finishes at the end of adolescence (Di Cristo, 2007). In mice, immature interneurons marked by DLX5 expression are observed until post-embryonic day 18 (P18), and most of interneuronal migration occurs between embryonic day 12 (E12) and P7 (Métin et al., 2006). In stark contrast to cortical projection neurons, which, like most CNS neurons, are born in the ventricular zone and migrate radially to populate the periphery of the telencephalon, cortical interneuronal neuroblasts are born in subcortical regions of telencephalon. They perform an elaborate migration from these subpallial regions to intermediate and marginal zones of the neocortex (IZ and MZ, respectively). MZ apically and IZ basally surround the cortical plate (CP), the region that eventually forms the six cortical layers. Interneuroblasts tangentially migrate through IZ and MZ and eventually radially invade the CP. In CP, interneuroblasts slow their migration and eventually mature - become sedentary, arborize, and form functional synapses with local projection neurons and other interneurons. In early postnatal development, interneurons selectively sort between cortical layers, depending on cell type and developmental origin (Miyoshi and Fishell, 2011).

The subpallium consists of five major regions: lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), caudal ganglionic eminence (CGE), septum (SE), and the preoptic area (POA). The MGE and LGE form the bulk of subpallium rostrally, while CGE does caudally; POA is a telencephalic region ventral to MGE. Most interneurons are born within MGE (60%) and CGE (30%), the rest arising predominantly from POA (Chu and

Anderson, 2015). SVZ-generated neurons MGE is the genic zone for most PV and STT interneurons. Within the MGE, majority of PV-expressing interneurons are born ventrally, and SST-expressing interneurons dorsally. Most 5HT3aR-expressing interneurons are born in the CGE (Jovanovic and Thomson, 2011; Rudy et al., 2011). An exception to the subpallial interneuronal origin is a small number of 5HT3aR-labeled interneurons in the subventricular zone of neocortex during development that persists to a small degree into adulthood, being responsible for adult neurogenesis in the neocortex (Dayer et al., 2005; Inta et al., 2008). Although some SVZ-derived neurons perform distant migrations to other brain regions (Ihrle and Álvarez-Buylla, 2011), SVZ-derived neocortical neurons in adulthood do not migrate far from their site of birth (Dayer et al., 2005).

A spatial and temporal expression of transcription factors determines interneuronal fate. Initially, interneuron progenitors commit to interneuronal fate by Mash1 and Dlx1/2 expression. As they exit the cell cycle, they also begin to express Dlx5/6. The development of MGE-derived interneurons depends on Nkx2.1 driving the activity of Lhx6. Exact transcription factors determining CGE interneuronal fate are not well established, in part given to the heterogeneity of interneuronal types CGE generates. Unlike MGE, CGE neuroblasts do not express Nkx2.1 and Lhx6, but they do express Dlx1/2 and Dlx5/6. So far, homeobox genes Gsx1 and Gsx2, Nkx6.2, and CoupTF1/2 are known to be required for specific CGE interneuronal types (Chu and Anderson, 2015; Luhmann et al., 2015; Wonders and Anderson, 2006).

## Interneuroblast Chemotaxis and Motogenesis

A combination of chemorepulsive and chemoattractive cues guides the migration of interneuroblasts along their tangential trajectories. Within the pallidum, Slit/Robo signaling also acts in chemorepulsion, as well as generation of new neurons within the MGE and their chemorepulsion from the MGE itself (Marín, 2013; Marín et al., 2003).

EphrinA5 signaling through the EphA4 receptor and RGMa repulse interneuroblasts from the ventral zone of the ganglionic eminence (O'Leary et al., 2013; et al., 2008). Membrane-bound CRD-neuregulin-1 signals through ErbB4 receptor to direct migratory interneurons to the cortex (Fisahn et al., 2009; Flames et al., 2004; Yau et al., 2003). In-

teraction of Sema3A and Sema3F with neuropilin receptors and Robo1 receptors keeps interneuroblasts from entering the striatum, and striatal chondroitin sulfate carrying proteoglycans synergize and spatially restrict this repulsion (Zimmer et al., 2010). Within the cortex, SDF(Cxcl12) through the Cxrc4 and Cxrc7 receptors attract interneuroblasts to the two migratory streams within MZ and IZ (Stumm et al., 2003; Sánchez-Alcañiz et al., 2011; Tiveron et al., 2006). Diffusible Ig-neuregulin-1 also attracts interneuroblasts to the migratory streams and directs tangential migration (Flames et al., 2004). Repulsive EphrinB3 and EphA4 signaling keeps migratory streams distinct, restricting migratory interneuroblasts to their respective tangential migratory zones (Zimmer et al., 2011). Netrin-1 signaling can also promote migration in a subset of MGE-derived interneuroblasts, but evidence is complex and conflicting (Marín, 2013; Marín et al., 2003; O’Leary et al., 2013; et al., 2009). Most of these studies have been done with MGE-derived interneurons, and many factors guiding interneuronal migration have yet to be discovered. Although some mechanisms guiding the migration are universal, interneurons from different genic regions differ in their guidance cues (Marín, 2013). Less is known about migratory cues directing CGE-derived interneurons, but these cells, despite their heterogeneity, can be unitedly distinguished by 5HT3aR expression, and serotonin signaling has recently been shown to regulate their migration (Murthy et al., 2014). Additionally, cues guiding interneuronal

migration seem to differ significantly from pyramidal neuronal cues. For instance, reelin, a molecule important for radial migration of pyramidal neuronal progenitors that is heavily expressed in the MZ, is dispensable for both radial and tangential migration of interneurons (Pla et al., 2006).

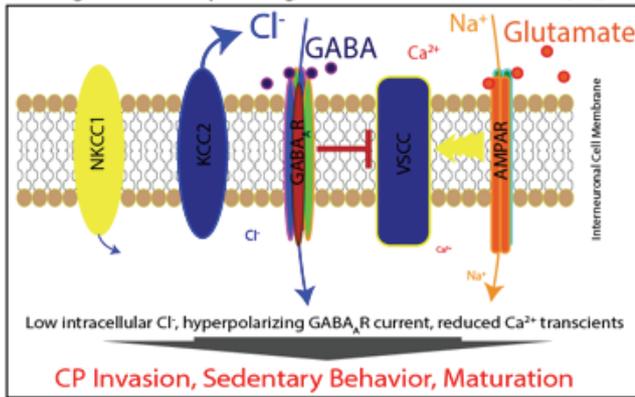
Simultaneously with guidance cues, motogenic cues promote migratory behavior of interneuroblasts and serve as signals of initiation and termination of migration. Multiple motogenic cues have been found: brain-derived neurotrophic factor (BDNF) through TrkB receptor, neurotrophin-4 (NT4), glial-derived neurotrophic factor (GDNF) signaling through GFRa1, and hepatocyte growth factor (Levitt et al., 2004; Polleux et al., 2002; Pozas and Ibáñez, 2005). However, though the aforementioned studies include *in vivo* experiments, the main experiments have been performed *in vitro*, and the *in vivo* role of these factors is unclear in light of dispensability of some of their receptors for interneuronal migration, such as TrkB and MET (receptor of hepatocyte growth factor), and other complications (Marín, 2013). Dopamine receptor activation also influences interneuronal migration: activation of D1 receptor promotes, and activation of D2 decreases interneuronal migration *in vitro*, with *in vivo* corroboration in knockout and overexpression studies (Crandall et al., 2007). Along these cues, GABA signaling is believed to play a central role in promoting interneuroblast migration, and regulating the termination of this migration and CP invasion.

## GABAergic Signaling of Interneurons

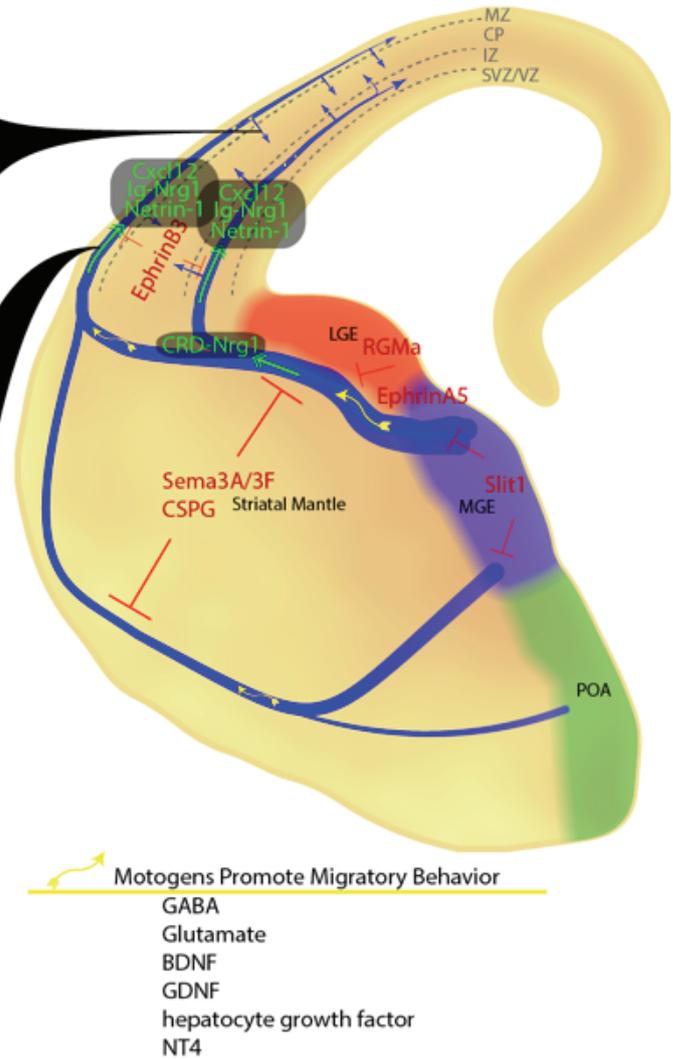
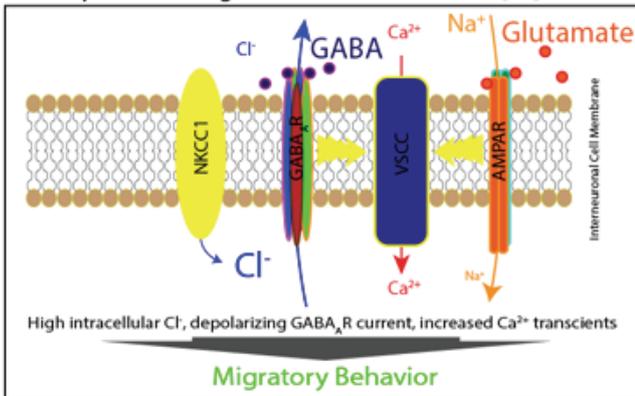
GABAergic signaling is not only an interneuron’s output into a neural circuit, but also a signal to the interneuron itself, both in autocrine and paracrine fashion, that serves as a neurotransmitter in maturity, and as a guidance or motogenesis cue in development. GABA is a ligand of GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs), which are ligand-gated Cl<sup>-</sup> channels, and metabotropic GABA<sub>B</sub> receptors (GABABRs), which are G-Protein Coupled Receptors (GPCRs) that act through G<sub>i/o</sub>. One of main actions of G<sub>i/o</sub> is activation of G-protein activated inwardly rectifying K<sup>+</sup> channels (GIRKs) (for a detailed discussion of GABA<sub>B</sub>Rs coupling to ion channels, view

(Padgett and Slesinger, 2010)). Mature interneurons express both of these receptors. Opening of either Cl<sup>-</sup> or K<sup>+</sup> channels in mature adult neurons is hyperpolarizing due to low intracellular [Cl<sup>-</sup>] and high intracellular [K<sup>+</sup>], making GABAergic signals hyperpolarizing through both GABA<sub>A</sub>Rs and GABA<sub>B</sub>Rs, resulting in inhibition of excitability. However, in development, high neuronal intracellular [Cl<sup>-</sup>] results in depolarizing GABA<sub>A</sub>R currents. This phenomenon is unique to GABA<sub>A</sub>R currents, as GABA<sub>B</sub>R-activated GIRK currents are consistently hyperpolarizing because K<sup>+</sup> mediates them.

GABA signals the stop of migration when intracellular  $[Cl^-]$  is LOW



GABA promotes migration when intracellular  $[Cl^-]$  is HIGH



**Figure 1.** Migration of interneuroblasts from interneurogenic subpallial zones to the neocortex relies on multiple guidance cues and motogens. Migratory streams of interneuroblasts in a cartoon model of E14.5 mouse brain are shown in blue. Motogens promote migratory behavior (yellow arrows, listed below slice), while guidance cues designate the direction of migration through chemorepulsion (red t-lines) and chemoattraction (green arrows). GABA, signaling through  $GABA_A$ Rs, serves as a strong regulator of interneuroblast migration. Its property as a motogen or a stop signal depends on intracellular  $[Cl^-]$ , which changes before neuroblasts invade the CP due to upregulation of KCC2. Thus, GABA can cue the CP invasion and maturation of individual interneuroblasts within a migratory stream while promoting the tangential migration of their neighboring interneuroblasts. Refer to the body of the manuscript for cue receptors, cited literature, and protein/signal acronyms. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; POA, preoptic area; neocortical structures: MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone.

## GABAergic Regulation of Interneuronal Migration

GABAergic signaling has been proposed to be the main mechanism regulating the timeline of tangential IZ and MZ migration, the decision to invade the CP, and subsequent maturation of interneurons (Bortone and Polleux, 2009), recently reviewed by Luhmann et al, 2015. According to this model, interneuroblasts perceive GABA signals through  $GABA_A$ Rs. The activation of  $GABA_A$ Rs can

either depolarize or hyperpolarize the neuronal membrane, depending on the direction of  $Cl^-$  ion flow, which depends on the intracellular  $[Cl^-]$ . Migratory interneuroblasts primarily express Na-K-Cl cotransporter 1 (NKCC1), which maintains a high internal  $[Cl^-]$  and depolarizing GABA responses. Depolarization of the interneuroblasts activates L-type voltage-sensitive  $Ca^{2+}$  channels

(VSCC), promoting migration through motogenic  $\text{Ca}^{2+}$  transients. During maturation, the neuroblasts downregulate NKCC1 and begin expressing K-Cl cotransporter 2 (KCC2), transitioning to low intracellular  $[\text{Cl}^-]$  and hyperpolarizing GABA responses. Expression of KCC2 serves as the switch from interneuronal migration to CP invasion and maturation by hyperpolarizing the membrane and reducing the VSCC-mediated  $\text{Ca}^{2+}$  transients.

Multiple lines of evidence support this model. GABA is well known for its trophic effects on cell growth, migration, synapse formation (Heck et al., 2007; Represa and Ben-Ari, 2005) and chemotaxis (Bouzigues et al., 2010; Heck et al., 2007) during development. Initial currents in a developing cortex are mainly GABAergic, tonic, and extrasynaptic, causing giant depolarizing potentials (GDPs) (Ben-Ari et al., 2007). Neuroblasts and mature interneurons express GABAARs, and ambient GABA (around  $.5 \mu\text{M}$ ) is found along their migratory path in MGE, IZ, and MZ (Cuzon et al., 2006). Interneuronal neuroblasts develop slow tonic extrasynaptic currents indicative of GDPs during migration through the MZ and IZ (Cuzon et al., 2006). Cortical cultures show deficient interneuronal migration when incubated with a GABA antagonist bicuculline, but migrate more robustly when a positive allosteric modulator of GABAARs diazepam is used (Cuzon et al., 2006). Cultured interneurons stop migrating upon KCC2 expression and show increased migration in KCC2 knockdown, which is dependent on VSCC-mediated  $\text{Ca}^{2+}$  transients (Bortone and Polleux, 2009). Additionally, concomitant glutamatergic signaling is required for these  $\text{Ca}^{2+}$  transients and migration (Bortone and Polleux, 2009). Inada et al., 2011 corroborated this model in vivo in P0-P3 mice, observing tangential cortical interneuronal migration in the MZ, and initiation of KCC2 expression upon CP invasion. This migration was reduced if GABAergic signaling was disrupted by either a GABA antagonist bicuculline, a heterozygous knock out of GABA-synthesizing enzyme GAD67, or reduction in intracellular  $[\text{Cl}^-]$  using a NKCC1 blocker. If the

effect of depolarization on intracellular  $[\text{Ca}^{2+}]$  was blocked by the  $\text{Ca}^{2+}$  chelator BAPTA, migration came to a halt (Inada et al., 2011). Cumulatively, these findings support the model proposed in Bortone and Polleux, 2009 of summative depolarizing glutamatergic and GABAergic currents during interneuronal migration, but antagonistic depolarizing glutamatergic and hyperpolarizing GABAergic currents upon KCC2 expression, which reduce VSCC-mediated  $\text{Ca}^{2+}$  transients, terminate migration, and initiate maturation.

Several other signals feed into this model to promote interneuronal migration. Taurine, an endogenous partial agonist of GABAARs, has elevated production in development, causes tonic GABAAR currents, and regulates the CP invasion of interneuroblasts (Furukawa et al., 2014). Signaling through GABABR also plays some role in interneuroblast CP invasion, but this role is indirect and unclear, since migrating interneuroblasts do not express GABABRs (reviewed by Luhmann et al., 2015).

Although the model has strong support, it needs further research and development. Direct measurements of currents and membrane potential changes that are responsible for interneuronal migration and CP invasion are limited. Performing such measurements is necessary to provide direct evidence of how interneuronal neuroblasts develop their interaction with the surrounding environment. In light of the recent implication of taurine, the contributions of GABA and taurine to GABAAR-mediated currents causative of tangential and radial (CP invasion) interneuroblast migrations need to be distinguished. Additionally, the mechanism guiding differential interpretation of GABAAR ligands by the interneuroblast still has to be explored from the side of differential GABAAR subunit expression, on which the subsequent section touches. Thus, although GABAergic regulation of interneuroblast migration is an active hypothesis in the field, direct supportive evidence is still needed and further mechanistic details have yet to be distinguished.

## Potential Regulation of GABAergic Currents by Varying GABA<sub>A</sub> R Subunit Expression

The character of GABAergic currents that an interneuron experiences throughout its migration and maturation depends on progressive changes in GABAAR subunit composition. GABAARs are heteropentameric receptors generally composed of 2 $\alpha$ , 2 $\beta$ , and a  $\gamma$  or  $\delta$  subunit, with 19 total subunits known (Olsen and Sieghart, 2008). The  $\alpha$  subunit composition in particular affects receptor sensitivity, desensitization, and deactivation properties, greatly varying current responses to GABA (Picton and Fisher, 2007). Additionally, GABAAR subunit composition affects whether the receptor localizes to a synapse. Typically, the sensitivity and kinetics imparted by subunits compliment the respective demands of synaptic and extrasynaptic localization. Extrasynaptic GABAARs require low desensitization to maintain a tonic current and typically include  $\alpha$ 4,  $\alpha$ 5, or  $\alpha$ 6 subunits; meanwhile, synaptic GABAARs include  $\alpha$ 1,  $\alpha$ 2, and  $\alpha$ 3 subunits (Belelli et al., 2009; Olsen and Sieghart, 2008). The stoichiometry of  $\gamma$  and  $\delta$  subunits in GABAAR composition imparts differences in receptor desensitization and subcellular localization, mediating mainly  $\gamma$ -dependent phasic synaptic and  $\delta$ -dependent tonic extrasynaptic currents, except for tonic extrasynaptic  $\alpha$ 5 $\beta$  $\gamma$  GABAARs (Olsen and Sieghart, 2009). The  $\beta$  subunits do not appear to influence intracellular localization, and impart subtle differences in sensitivity and channel kinetics that are less well characterized than  $\alpha$ ,  $\gamma$ , and  $\delta$ .

Cortical GABAAR subunit expression in adulthood predominantly consists of the  $\alpha$ 1,  $\beta$  (2,3),  $\gamma$ 2, and  $\delta$  subunits, though there are big differences between layers, and  $\alpha$ (2-5) are also expressed at lower levels (Hörtnagl et al., 2013). However, this expression changes throughout development and age (Fillman et al., 2010; Yu et al., 2006), and varies by region. Namely, the developing cortex has a reduced expression of  $\alpha$ 1 and  $\delta$ , and an increased expression of  $\alpha$ 3 and  $\alpha$ 5 (Laurie et al., 1992). During interneuronal migration, MGE expresses  $\alpha$ (3,4,5),  $\beta$ (1-3), and  $\gamma$ 1 subunits; the neocortex addition-

ally expresses  $\alpha$ 1,  $\gamma$ (2,3) and does not express  $\beta$ 2 (Cuzon et al., 2006). These regional and temporal expression differences do not show directly interneuronal GABAAR subunit composition, but hint to the developmental changes of the regions as a whole. In particular,  $\alpha$ 1 subunit, that is associated with mature synapses, is expressed later in development, while  $\alpha$ 3 expression is enriched developmentally. In addition to developmental enrichment,  $\alpha$ 3 subunit RNA does not developmentally undergo editing that is widespread in adulthood. The low desensitization kinetics of this unedited  $\alpha$ 3 subunit allow it to summate phasic responses to high concentrations of GABA, as may be encountered in migration and synaptic maturation by axonal growth cones (Rula et al., 2008). This puts the changing expression of  $\alpha$  subunits as a good candidate for the mechanism of regulating the characteristics of GABAergic currents in a developing interneuron.

Exact measurement of GABAergic currents and GABAAR subunit expression in migrating interneurons needs to be further characterized. So far, it's known that the amplitude of currents in response to GABA application in migrating interneurons significantly increases as they cross from MGE into the neocortex. Semiquantitative assessment of individual interneuronal GABAAR subunit expression by reverse transcription PCR correlates this transition with increased expression of  $\gamma$  and  $\alpha$  subunits, especially  $\alpha$ 1. This coincides with increased benzodiazepine sensitivity of GABAergic interneuronal currents, indicating a greater  $\alpha$ (1,2,3,5) $\beta$  $\gamma$  component (Carlson and Yeh, 2011). It is possible that these changes correspond to a transition from tonic currents to a cell with active axonal processes that uses phasic currents for migratory guidance and synaptogenesis. However, further experiments quantifying interneuronal GABAAR subunit expression at different migratory timepoints, the extent of subunit-specific currents, and the impact of loss of function of these currents on interneuronal migration still need to be examined.

## Clinical Significance

In assessing the significance of a proper orchestration of interneuronal development, it is important to consider the grave clinical consequences of interneuronal developmental defects. Indeed, defects in formation of GABAergic cortical circuits due to improper interneuronal migration, arborization, maturation, or synaptogenesis can impair proper cortical signaling, cause imbalance of excitatory and inhibitory signals, and result in hyperexcitability. Pathologically, this can result in a host of neuropsychiatric disorders: epilepsy (Bozzi et al., 2012), schizophrenia (Volk and Lewis, 2014), autism spectrum disorders (Dani et al., 2005), (Belmonte et al., 2004), bipolar disorder (Uribe and Wix, 2012), and others. Pathogenic interneuronal migration typically occurs during development,

but can also occur in adulthood, such as epilepsy in conjunction with abnormal migration of new neurons in hippocampus following brain trauma (Shetty, 2014). For this reason, improving our understanding of the signals and mechanisms guiding neocortical interneuronal migration improves our understanding of disorder pathophysiology, therapy development, and the limitations of adult interneurogenesis. In addition to finding therapy for aberrant migration, this includes therapy for repopulating circuits deficient in interneurons, such as cortical interneuron deficiency in epilepsy after traumatic brain injury (Avramescu et al., 2009). Implantation of interneuronal stem cells into cortex (Southwell et al., 2014) and hippocampus (Shetty, 2014) is currently being developed in rodents.

## Concluding Remarks

Currently, a rich interneuronal diversity is known. These interneurons all secrete GABA, have local axonal projections, and originate subpallially. However, past these commonalities, their morphological, physiological, and cytochemical properties are richly different, indicating a large variety of different circuit functions. Functional grouping of interneurons is currently underway with some clearly distinguished types and multiple undefined or vaguely defined types. Further systematization is needed, which in large relies on progress in designing universal terminology and nomenclature. Developmental processes that place these interneurons into their adult niches are known generally, but need further systematization and research. Since there are different interneuronal types that arise from different subpallial regions, the guidance cues are not always universal to all interneurons. The specific neurogenic niches of each interneuronal type are less well known, and even less known are mechanisms behind specifying these niches. Evidence for multiple migratory cues and motogens has been presented, but much of it is *in vitro*, so the actions of these cues

*in vivo* have yet to be explored, or have presented contradictory evidence. Additionally, new cues are still being discovered, necessitating their incorporation into present models. Thus, the significance of each cue in mediating interneuronal migration still needs further exploration. Nonetheless, the vital role of GABAergic signaling in regulation of interneuronal migration and maturation is supported by substantial evidence. This type of regulation is unifying of all interneurons, as they all secrete and sense GABA. The GABAergic mechanism of controlling migration, however, needs further research and *in vivo* support. This in particular refers to accurately assessing the time points of regulation of GABAergic currents by changes in KCC2 and GABAAR subunit expression. Given these details, it will be possible to present interneurons with a migratory environment and confirm their proper response to the surrounding cues. In the meantime, current knowledge presents high significance to interneuronal migration both from clinical and basic science viewpoints, but the ability to explain and affect this developmental process is limited.

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