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LETTER FROM THE EDITORS

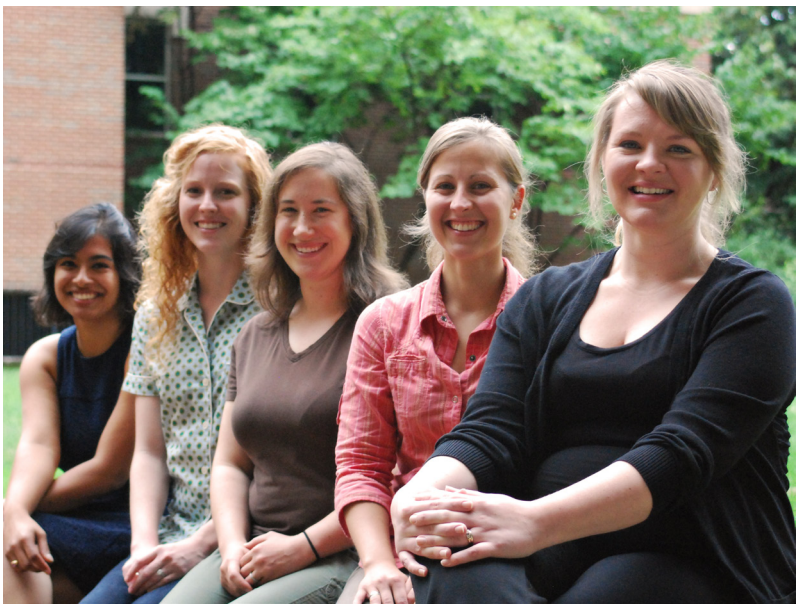
Dear Friends and Colleagues of the Vanderbilt Neuroscience Community,

The *Vanderbilt Reviews Neuroscience* is still alive and kicking, and we're excited to bring you another exciting issue this year! With preserving our recent style and formatting makeover, we hope to promote this layout as the permanent template for all future issues. A new feature of this year's issue is our wonderful cover art, a scientific homage to the cartoonist style of one of our favorite publications, *The New Yorker*. Special thanks to Eva Sawyer for her artistic (and scientific) talents! We also included a new section in this issue called "Grad Student Corner" that is dedicated to current student issues and potential resources that may be valuable to our students. Regardless, our goal remains the same in continuing to highlight the talented writing and research interests of the newest Ph.D. candidates in the Vanderbilt Neuroscience Program. This issue features twelve candidate reviews covering topics from both systems and molecular neuroscience, including: the role of neurotransmitter transporter regulation in diseases; circuit processing in anxiety and autism, and the role of the developing amygdala in childhood maltreatment; circuit refinement and neurotransmitter signaling during development; touch mechanisms; and new potential CNS drug targets, as well as a patient-derived stem cell model for Parkinson's disease. We also highlight a collection of first-author papers published by Vanderbilt Neuroscience students over the past calendar year. Our students excel in publishing in high impact journals, making insightful contributions that are highly regarded by external peer review. Keep up the good work, and thanks for making our jobs easier by providing such great content for the *VRN*!

This *VRN* issue would not have been possible without the painstaking contributions from our tenacious editing board. Our Associate Editors, Suzanne Avery, Hayley Clay and Barbara O'Brien (all pictured below), are incredibly talented and hardworking. For the first time, our associate editing board consisted of all females (girl power!), and the future of *VRN* remains bright under their leadership for next year. We had a blast with you guys! We would also like to recognize the outgoing Co-Editors-in-Chief, Drs. Andrew Hardaway and Maureen McHugo, as well as Founder/Editor-in-Chief, Dr. Chris Ciarleglio, for helping establish the *VRN* and equipping us with a valuable network of resources to successfully produce this year's issue. Our sincerest thanks also go to both Drs. Mark Wallace and Doug McMahon, who have helped to guide the role of the *VRN* in sculpting each qualifying student's graduate experience. Their support and enthusiasm for a student-run endeavor like the *VRN* is only one example of the Vanderbilt Neuroscience Program's ideals in promoting the development of successful, independent thinkers. Finally, we would like to thank the 2012 qualifying class for their hard work in generating the true foundation of this issue and tolerating our many edits and emails. This would not have been possible without you!

Your Co-Editors-in-Chief,

Sudipta Chakraborty and Juliane Krueger Fister



From left: Sudipta Chakraborty, Hayley Clay, Barbara O'Brien, Juliane Krueger Fister, and Suzanne Avery

Juliane Krueger Fister and Sudipta Chakraborty

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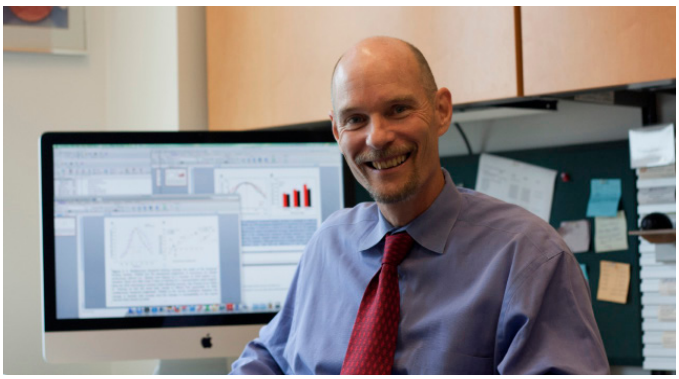
Vanderbilt Reviews Neuroscience(VRN) is an open-access journal (http://vrn.vanderbilt.edu). *VRN* is the official journal of the Vanderbilt University Neuroscience Graduate Program and the Vanderbilt Brain Institute. *VRN* is a collection of reviews submitted by Vanderbilt Neuroscience Graduate Students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

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



A Message from the Director of the Vanderbilt Brain Institute

It is with a sense of deep pride that I review this fifth issue of *VRN*. Once again I am very impressed with the quality and diversity of the reviews that make up this volume, as well as with the synopses that illustrate the exceptional year our trainees have had in the realm of scholarship.

This past month has seen the VBI staff assemble the proposal for renewal of our training grant, and in gathering the materials necessary to make the strongest possible arguments for continuing (and increased) support, I was pleased to be able to point to the numerous successes that we have had over the past several years. Along with continuing our exceptional record of scholarship, with numerous papers in high profile journals authored by our trainees, we have had a remarkable record of success in garnering extramural support in the form of individual fellowship grants and foundation awards. Past students of our program are now transitioning to faculty positions at prestigious institutions such as Harvard, Yale, and Duke (and Vanderbilt of course!), and our newly minted PhDs continue to do postdoctoral fellowships in some of the world's leading neuroscience laboratories. With these accomplishments to reflect on, I feel confident that our mission to train the future leaders in the neurosciences is well on track!

Yours in science,

Mark T. Wallace, PhD

A Message from the Neuroscience Program Director of Graduate Studies

A neuroscience journal run, edited and contributed entirely by graduate students - how cool is that? Plenty, when the quality of the work published and the care with which it is assembled is as special as *VRN*. *VRN* showcases the scholarly work of our outstanding students as they assemble and synthesize their knowledge base and move toward fully implementing their dissertation research. The original articles highlight the insight and intellectual curiosity of Neuroscience graduate students at Vanderbilt and foreshadow their novel contributions to solving the mysteries of the brain. Each issue is a special pleasure to behold.

As ever,

Douglas McMahon, PhD

An Update from the Neuroscience Student Organization President

First, I would like to welcome all incoming students to our wonderful Neuroscience community. Be sure to get to know your new fellow graduate students as we will be each other's future peers and colleagues. Second, I want to congratulate our twelve new PhD candidates, whose qualifying reviews are featured in this issue. This group of talented young scientists is now moving on to phase II of the qualifying process, which includes a NRSA-style research thesis proposal.

As the Neuroscience Student Organization (NSO) president it is my distinct honor to highlight here some of the achievements of our student body. We currently have 14 active Ruth L. Kirchstein National Research Awardees, as well as one award each from Autism Speaks, the National Science Foundation and the American Heart Association. Furthermore, since the last VRN issue, 10 students have successfully defended their PhD and moved on to bigger and better things. This year, we have a total of seven Clinical Neuroscience Scholars (CNS) Program recipients. The CNS program is now in its second year and has been incredibly successful in connecting scientist and medical doctors to bridge the gap between the basic and clinical research sciences. Last year, we all had the pleasure to listen to the CNS scholars' experiences during a series of CNS research forums.

A big thanks goes out to the organizers of our Neuroscience Boot Camp. This boot camp has now taken place for two years in a row and has been a tremendous success in connecting the incoming students, as well as providing senior graduate students with teaching experiences and creating an informal atmosphere to discuss fundamental neuroscience topics. Jeff Jones, Terry Jo Bichell, and Ben Dean were instrumental in developing and executing a curriculum for this camp, which was held one to two weeks prior to the first day of classes. Our student body comes from diverse educational backgrounds, and the boot camp is designed to bring the students together on the same level as they enter the program. Lectures and paper discussions are prepared by upper level students, and incoming students are encouraged to participate by drawing on a whiteboard as they would during the qualifying exam process. Overall, I believe the camp has been a great success and I truly hope it will be continued in the upcoming years.

Next, I would like to point out a few changes to the Neuroscience curriculum. In order to better streamline classes, our fundamental neuroscience courses, Systems Neuroscience and Cell and Molecular Neuroscience, are now called Fundamentals I and II and are building upon each other and further integrate the dependency of molecular and systems neurosciences on each other. A host of elective courses have also been added to meet the demands of the student body as well as the ever-growing field of neuroscience. This year, we also have our inaugural class of graduate students through the Educational Neuroscience track. I am looking forward to their progress and contributions to science and educational research.

Another yearly staple is the student run Neuroscience Retreat. The 16th annual retreat will be held again at the Joe C. Davis YMCA Outdoor Center. This year our goal is to promote interactions between new and current students as well as the overall neuroscience community. The retreat will include short talks from Dr. Kim Petrie and Dr. Ruth Schemmer regarding important concepts for early, mid and later career development, including topics ranging from effective communication, resume writing do's and don'ts, and opportunities for intern and externships here at Vandderbilt. We will also get a first-hand account of what it is like to start a lab from a Vanderbilt graduate, Dr. Brad Greuter. A poster session open to students, staff and post-docs will be held to allow the Neuroscience community to get a taste of the exciting science occurring on campus. We are also happy to host NIH Distinguished Investigator Dr. Kenneth Fischbeck, who will give the Keynote lecture.

Lastly, I will echo my predecessors, and encourage everyone to get involved in any facet of our student organization and neuroscience community. We are an organization of students for students and can only grow with your support and commitment.

Yours truly,

Juliane Krueger Fister

OUTREACH+ EDUCATION

This year's **Brain Blast** was held Saturday, March 2, and children from the community were introduced to hands-on neuroscience research with graduate students, post-doctoral fellow, and principal investigators.

During Brain Blast, children explored brain research by experiencing optical, somatotopic, and sensorimotor illusions with the Tong and Wallace labs. They used their own brain waves to control objects with the Roe Lab's MindFlex demonstration, extracted DNA from strawberries with the Emeson Lab, and touched a real human brain at the Tick-Tock station! Other labs brought animals so that the community could learn about how we use animal models to investigate different aspects of brain function. The circadian group brought their Madagascar hissing cockroaches, and many children (including our very own Jeff Jones) loved handling the creepy crawly critters. Less hands-on, Eva Sawyer and Duncan Leitch from the Catania Lab brought an American alligator to show the highly sensitive receptors lining the jaw of this fantastic reptile. Children even had some microscope time with Ben Dean from the Gamse Lab looking at fluorescent neurons in transparent fish. Other hands-on activities also encouraged interest in brain research. Student-run booths included putting together 3-D brain puzzles, building neurons, making brain hats, or guessing objects via smell, sound, or touch. Be sure to keep a look out for updates on next year's Brain Blast.

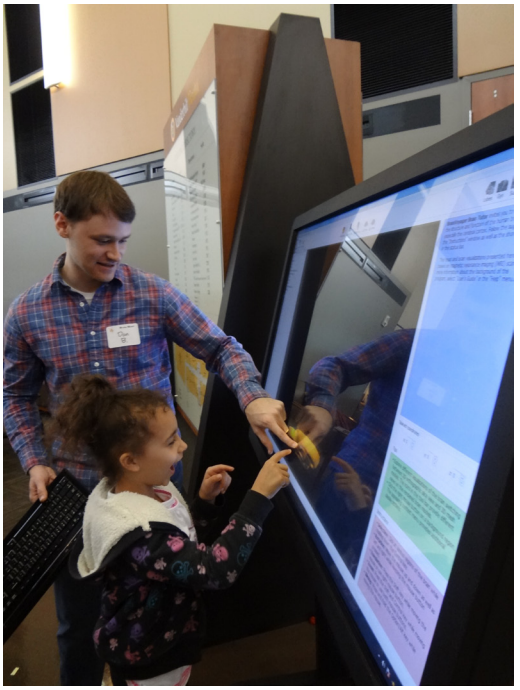
Top right: Brain Blast booths at One Hundred Oaks Mall.

Middle right: Jeff Jones showing off a Madagaskar hissing coakroach.

Bottom right: A very interative touch screen was a big hit with the audience, demonstrated here by Dan Bermingham.

Top left: A little girl very obviously enthralled with the Catania lab alligator.

Bottom left: Loui, Terry Jo Bichell's son checking out the zebrafish through the microscope with help from Ben Dean.



** photos provided by Barbara O'Brien and Eva Sawyer*

A Message From Your Middle Tennessee SfN Chapter

Dear Members of the Vanderbilt Neuroscience Community,

Thanks to you, it has been another exciting and successful year for the Middle Tennessee Chapter of the Society for Neuroscience. Our chapter is one of well over 100 spanning the globe and is gaining increasing attention from the national organization for its dynamic character and exciting initiatives. Last year we were invited to give a presentation on some of our innovative activities (such as our pumpkin decorating social) at the Chapters Workshop at the Annual Society for Neuroscience meeting.

Since its inception more than 10 years ago, our chapter has continued to grow and expand its membership beyond Vanderbilt to include a number of local institutions such as Meharry, Austin Peay, Fisk and TSU. At this year's Annual Meeting, faculty from several of these schools treated us to short talks about their research, followed by our Data Blitz presentations by local trainees and a social jointly sponsored by the Neuroscience Student Organization. We just completed our 3rd Summer Enrichment Research Program in Education and Neuroscience Training, or SERPENT, with a student from TN Technical University, Matthew Defenderfer. This program allows an undergraduate from a local institution to come to Vanderbilt and do research over the summer. It also includes an outreach component, since the summer student is tasked with developing a brief educational lesson/experience for the public or local K-12 schools. Matt spent his summer working in Mark Wallace's lab and for his outreach project he developed an interactive optical illusion game that we will highlight at next year's Brain Blast. Matt will be applying to Vanderbilt's PhD program in neuroscience after graduating next year. This would be our 3rd student to matriculate into a graduate program, making our rate of SERPENT students who continue on to advanced research 100%!

If you are not a member of the chapter already, I would strongly encourage you to join us and become an active part of the local neuroscience community. Being part of the chapter increases your networking opportunities, provides a forum for sharing scientific ideas, allows you to engage in educating the public about neuroscience research, and there are several travel and lecture awards available. Of course, there are fun activities as well (recall the balloon "flocking" of Mark's office). What the chapter is and what it does is determined by its membership. It is the goal of the current leadership team to make the Middle TN Chapter a showcase example of what can be done through the local chapters. At present, we continue to focus on expanding our membership through increasing the involvement of other local institutions and developing new funding mechanisms so that we can create innovative and novel programming initiatives.

Do not miss the opportunity to be a part of a vibrant and dynamic community of neuroscientists right here in your neighborhood! We can't realize the full potential of our chapter and make it into the national treasure that we aspire to without your involvement and support. You are just one click away from joining and/or making a donation! Don't wait, do it now! (http://www.mtncsfn.org/).

Bruce D. Carter, PhD

President of the Middle Tennessee Chapter of the Society for Neuroscience

Modern Science Chaos: Taking New Initiatives

The changing climate of today's modern science world has resulted in a unique shift in career paths and job outcomes around the country. According to the NIH Biomedical Workforce Working Group Report, the number of Ph.D. trainees has significantly increased since 2004, in parallel with the doubling of the NIH budget. This allowed for an increase in the number of predoctoral research grants, whereas placement on training grants were previously a major source of Ph.D. recruitment. Unlike training grants, individual research grants do not necessarily require the same level of training that is specific for professional development. Concurrently, universities began to put a focus on increasing the number of non-tenure-track positions that are dependent on outside funding and would not require the university to cover their salaries. Together, this brews the perfect storm of chaos: the increased production of Ph.D.'s only trained to excel in the academic sector, in an environment where there are not nearly enough positions available for the majority of them.

Therefore, a strong demand exists for better training PhD candidates for "non-traditional" career routes outside of academia. Clearly, the focus of Ph.D. trainees remains to successfully complete thesis projects and learn to become successful, independent scientists. However, while some programs may require teaching experiences for graduation, a strong absence of exposure to any other career paths is highly evident - students are only trained to conduct and communicate research, apply for funding, direct and maintain projects, and perhaps teach to a minor degree. With the current state of science, this proves to be disadvantageous to the biomedical field, as candidates enter alternative career paths without any previous training or necessary knowledge to become successful in these sectors. Yet, Ph.D. trainees are able to critically question, evaluate and solve problems, making them highly marketable across many fields.

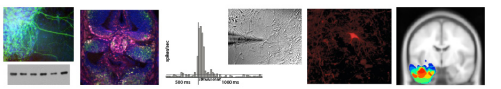
This rising concern is widespread, as even the National Science Foundation (NSF), a prominent scientific funding and research agency, put out a call this year for innovative ideas that would enhance STEM (science, technology, engineering, and mathematics) graduate education. Our own Neuroscience Student Organization (NSO) even put in an application of ideas (geared towards women and minorities in the STEM fields), in addition to other PhD students around campus. Vanderbilt's BRET (Biomedical Research, Education and Training) office is currently looking into revolutionary methods that may integrate training experiences into the graduate education system. We optimistically hope for a new system for future graduate students, where official Ph.D. candidates are able to participate in short-term, unpaid intern/externships that promote immersion into various sectors outside of academia that are actively part of modern science. In the meantime, I encourage you to reach out on your own towards sectors that may be of interest to you as a career path, be it consulting, scientific writing, regulatory affairs, biotechnology project management, clinical trials, etc. Speaking from personal experience, even sending a simple email to someone in your area of interest about their careers and their training experiences along the way can prove to be fruitful in both gaining new information and forming new professional contacts. In fact, Vanderbilt has several clinical research labs that can provide a wealth of information in terms of how this sector operates, in addition to the presence of other biotech, regulatory and writing agencies around Nashville. I also urge you to get involved on committees and programs around campus that pertain to your interests. There are so many to offer, including the Saturday Academy at Vanderbilt for the Young (SAVY) and Weekend Academy at Vanderbilt (WAVU) programs for promoting education for children in grades K-10; the Scientist Educator Program; the Editors' Club; the HHMI/ VUMC Certificate Program in Molecular Medicine (CPMM); and even our own VRN editing board. Until agreements develop between graduate programs and funding agencies concerning the implementation of new training experiences, we all need to "take the bull by its horns" and prepare ourselves the best we can for an exciting new wave of science careers that will impact the world in ways previous generations have never seen!

Sudipta Chakraborty

Resources for Neuroscience Students Compiled by Neuroscience Students:

Campus Resources

- Vanderbilt Research News: http://news.vanderbilt.edu/research
- Neuroscience Student Organization: http://www.mc.vanderbilt.edu/root/vumc.php?site=NSO
- Vanderbilt Depolarized (Student Blog): https://my.vanderbilt.edu/vanderbiltdepolarized
  BRET Office: https://medschool.vanderbilt.edu/bret
- Center for Teaching: http://cft.vanderbilt.edu
- Graduate School: http://www.vanderbilt.edu/gradschool
- Eskind Library: http://www.mc.vanderbilt.edu/diglib
- Vanderbilt CORES: http://www.mc.vanderbilt.edu/root/vumc.php?site=CFUIS
- SAVY/WAVU Teaching Programs: http://pty.vanderbilt.edu/contact/employment/for-vu-faculty-staff-and-students
- Vanderbilt Brain Institute: http://braininstitute.vanderbilt.edu/index.php
- Middle TN Chapter for the Society of Neuroscience (MTNCSfN): http://www.mtncsfn.org

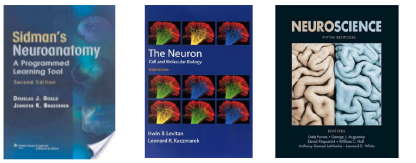


Find a references: Use Google Scholar scholar.google.com and make sure to enter Vanderbilt University Library so you can access journals directly.

Cite your reference:

- For Macs: www.papersapp.com/papers
- For PCs: www.mendeley.com

Share your documents: www.dropbox.com
Plan a meeting: www.doodle.com
Professional networks: www. linkedin.com and http://www.researchgate.net



Teach yourself molecular biology concepts through youtube:

- Central dogma: http://www.youtube.com/watch?v=J3HVVi2k2No
- Post-translational modifications: http://www.neb.com/nebecomm/tech_reference/epigenetics/epigenetics.asp#.UCZCAI7lpqg
- Vesicle dance: http://www.youtube.com/watch?v=BdKbRgT4hn8

Seriously teach yourself molecular biology concepts:

- NCBI main page: www.ncbi.nlm.nih.gov/About/index.html
- Basic Cell Biology: www.ncbi.nlm.nih.gov/About/primer/genetics_cell.html
- Great review lectures by noted scientists: www.ibioseminars.org

Find protocols for new experimental techniques:

- The protocol library: www.microbelibrary.org/about/51
- Wikipedia for methods: openwetware.org/wiki/OpenWetWare:How_to_join
- Protocol database: www.sciclips.com/sciclips/bio-protocols.do
- Current Protocols in Electrophysiology: http://www.sciclips.com/sciclips/bio-protocols.do?catName=Neuroscience&l2CatName=Electrophysiology
- See videos of techniques through The Journal of Visual Experiments: www.jove.com

Get help with calculations:

- Dilution calculator: www.tocris.com/dilutionCalculator
- Molarity calculator: www.graphpad.com/quickcalcs/Molarityform.cfm
- App calculator: http://www.sigmaaldrich.com

Neuroanatomy:

- www.brain-map.org
- http://mouse.brainarchitecture.org
- www.brainmuseum.org

RESEARCH HIGHLIGHTS

Overcoming Dravet syndrome by overcoming the GABRG2(Q40X) mutation

Xuan Huang, Tian, M, Hernandez, CC, Hu, N, & MacDonald, RL (2012). The GABRG2 nonsense mutation, Q40X, associated with Dravet syndrome activated NMD and generated a truncated subunit that was partially rescued by aminoglycoside induced stop codon read through, *Neurobiology of Disease* 48(1):115-123.

Epilepsy is one of the most common neurological disorders, and can arise from brain injury or genetic mutation. Commonly, mutations causing epilepsy occur in genes key to maintaining the electrical balance of neurons, including voltage-gated ion channels and $GABA_A$ receptors. In Huang et al. (2012), the Macdonald lab investigates a mutation seen in one of the more insidious types of idiopathic genetic epilepsy, Dravet syndrome. This form of epilepsy involves frequent seizures and progressive intellectual decline, and many cases can be traced back to nonsense mutations resulting in premature stop codons.

The mutation of interest in this study, GABRG2(Q40X), causes a premature stop codon the $GABA_A$ γ2S subunit. Human embryonic kidney (HEK 293T) cells were transfected with wild-type γ2S or γ2S(Q40X) and probed for changes in γ2S mRNA expression after knockdown of a component of the nonsense-mediated decay machinery. Many mRNAs containing the Q40X mutation were indeed degraded via nonsense-mediated decay, and those mRNAs that did make it to protein translation produced only a truncated peptide. This peptide did not mature, as evidenced by a lack of glycosylation, and was not expressed at the cell surface, as a wild-type γ2S subunit normally would be.

In addition to its problems with mRNA stability, protein expression, and protein maturation, γ2S subunits containing the Q40X mutation were not incorporated into $GABA_A$ receptors. HEK 293T cells transfected with the α1, β2, and γ2S(Q40X) subunits exhibited no γ2S(Q40X) surface expression, and the α1 and β2 subunits displayed similar surface protein expression patterns as cells that were transfected with α1 and β2 subunits but not γ2S. Assays for whole-cell receptor levels indicated that the lack of γ2S(Q40X) surface expression was due to an overall lack of γ2S(Q40X) expression, rather than a defect in its secretion to the cell surface.

Cells transfected with the γ2S(Q40X) subunit also displayed functional abnormalities consistent with their exclusion from $GABA_A$ receptors. Compared to wild-type, receptors on γ2S(Q40X)-transfected cells displayed reduced peak amplitudes in GABA-evoked currents and enhanced sensitivity to zinc-mediated inhibition. These phenotypes, however, matched those of cells expressing only the α1 and β2 subunits, indicating that $GABA_A$ receptors in cells transfected with γ2S(Q40X) were actually α1β2 receptors.

The antibiotic gentamicin can be used to promote read-through of premature stop codons in mRNA. Gentamicin treatment of cells transfected with γ2S(Q40X) had increased overall expression of the mutant γ2S protein. This phenotype was enhanced when the premature stop codon was changed to a TGA stop codon, which increased the cell's gentamicin-mediated read-through abilities. In addition to increasing overall protein expression of γ2S(Q40X), gentamicin treatment enhanced surface expression of γ2S(Q40X). These γ2S(Q40X) subunits at the cell surface were incorporated into functional $GABA_A$ receptors, as electrophysiological recordings subsequently showed two hallmarks of γ2S-containing $GABA_A$ receptors: zinc insensitivity and diazepam-mediated current amplitude potentiation.

These data not only provide insight into the mechanism of Dravet syndrome cases stemming from the GABRG2(Q40X) mutation, but also a mechanism by which the mutation's effects may be overcome. With the ability to partially rescue the mutant phenotype with premature stop codon read-through, this work provides a promising trajectory for future investigations into how to treat genetic forms of epilepsy.

He may be rough around the edges, but that reptile is highly sensitive

Duncan B Leitch, & Catania, KC (2012). Structure, innervation and response properties of integumentary sensory organs in crocodilians, *Journal of Experimental Biology* 215: 4217-4230.

Crocodilians may have thick skin, but that skin is more sensitive than your fingertips. The cover of the December 2012 issue of the Journal of Experimental Biology promi-

nently featured Duncan Leitch's article on somatosenory receptors of crocodilians, experts at detecting and catching prey. What gives them this incredible ability to so swiftly and precisely detect the exact location of their target? Leitch argues that it begins with the fine mechanoreceptors in the skin that are able to detect the slightest vibration at the water's surface.

The integumentary sensory organs (ISOs) visibly located on the skin of alligators and crocodiles have been speculated to serve many functions from sensing electric or magnetic properties of water to secreting protective oils. Leitch and Catania (2012) designed a study that would examine the distribution, structure, and physiological responses of ISOs for alligators and crocodiles to provide more insight into the function of these unique receptors. Using a behavioral paradigm, they ruled out the hypotheses of these being electro- or magneto- receptors and showed that somatosensory input alone is sufficient for the animal to locate its target.

While crocodiles have ISOs distributed on their entire bodies, ISOs on alligators are restricted to the head, more specifically in and around the mouth and jaw. Each ISO is composed of free nerve endings, discoid receptors, and terminals that appeared to be similar to the Paciniform corpuscles, all of which contribute to the ISO's extreme sensitivity. At the center of each ISO is a dermal Merkel cell column. To examine the sensitivity of this receptor, the authors recorded in vivo extracellular single- and multi-unit activity from the trigeminal ganglion while stimulating the skin with fine wooden probes or calibrated von Frey hairs. The smallest and most numerous receptive fields were located at the rostral portion of the face. Thresholds of ISOs varied, but in some cases, the threshold could not be recorded because it was smaller than the smallest calibrated force that could be applied. The most sensitive ISOs were found near the teeth and mandible. When a sea salt solution was applied to the receptors, no activity was detected. Similarly, activity was not detected in response to a 9V battery placed in the water. These results indicate that the receptors do not detect ionic changes in the water. Similar results were shown for recordings from the spinal nerves that innervated ISOs on the limbs and digits.

In examining the structure and function of ISOs, this article supports the hypothesis that ISOs are mechanoreceptors that specify the location of a disturbance in the water, based on the strength of the vibration signal. To correlate these findings with behavior, the authors recorded orientation to a food pellet dropped in water or to freely swimming fish. In complete darkness and with a white noise masker, crocodilians were able to orient to and efficiently capture prey, suggesting the sensitivity of ISOs plays a large role in identifying the location of a target, even when auditory and visual cues are absent.

The semantics of ecstasy use

Tristan J Watkins, Raj, V, Lee J, Dietrich, MS, Cao, A, Blackford, JU, Salmono, RM, Park, S, Benningfield, MM, Di Iorio, CR, & Cowan, RL (2013). Ecstasy (MDMA) polydrug users have altered brain activation during semantic processing, *Psychopharmacology* 227(1): 41-54.

Recreational experimentation with ecstasy (3,4-methylenedioxymethamphetamine or MDMA) has risen dramatically in the United States in recent years, with first-time users increasing 23% between 2008 and 2009. This trend is especially concerning due to the potential for long-term negative impact on the central nervous system—ecstasy use has been associated with both neuroanatomical and neurophysiological effects, such as degeneration of presynaptic axon terminals and dysregulation of serotonergic (5-HT) function, as well as long-term neurocognitive deficits, particularly within the realm of memory ability. Although long-term abstinence may be associated with partial improvement, several studies suggest sustained effects despite temperance. Given rising use and the potential for long-term negative impacts, it is critical that we develop a better understanding of the effects of ecstasy on the human brain.

The most consistent neurocognitive deficits found in ecstasy users have been in verbal memory, suggesting that verbal memory ability may be a sensitive marker of ecstasy-related impairments. As such, probing brain regions associated with verbal memory impairments in ecstasy users may be a useful approach for understanding the neural basis of ecstasy toxicity. Therefore, in a recent Psychopharmacology report, Tristan Watkins and colleagues examined the association of lifetime ecstasy use with semantic memory performance and brain activation. In this study, abstinent ecstasy users (n=23) and controls (n=11) performed a two-part semantic encoding and recognition task while acquiring functional magnetic resonance imaging (fMRI) data. During the encoding task, subjects mem-

orized both words and non-words (pronounceable Dutch words that would have no meaning for non-Dutch speaking subjects). During the recognition task, subjects were randomly presented with previously-shown words and non-words paired with phonological homophones that had not been previously shown, and asked to choose the prior studied word. Functional MRI data were collected during both parts of the semantic encoding and recognition task, and semantically-relevant brain activations were investigated using the contrast of word > non-word. During the encoding phase, ecstasy users had greater fMRI activity than controls in bilateral language processing areas, including Brodmann areas 7, 39, and 40. However, there were no between-group differences in semantic memory ability during the recognition task, nor were there differences in fMRI activity during recognition. These findings suggest that in order to achieve normal memory ability, ecstasy users' brains may use compensatory mechanisms, whereby greater neuronal activity during encoding is necessary to preserve verbal memory performance.

These conclusions, although preliminary, are in line with known effects of ecstasy-induced 5-HT toxicity in the brain. Ecstasy use is associated with degeneration of presynaptic axon terminals, 5-HT depletion, reduced binding to 5-HT reuptake transporters (5-HTT), reduced levels of 5-HT breakdown product 5-hydroxyindoleacetic acid (5-HIAA), and chronic upregulation of 5-HT2A receptors. Since the net effect of 5-HT in the cortex is inhibitory, reduced 5-HT cortical signaling in ecstasy users—whether from axon loss or due to chronic reductions in 5-HT signaling—would be consistent with increased cortical activation relative to control subjects. This study highlights potential consequences of ecstasy-induced 5-HT neurotoxic effects and will guide future studies in human ecstasy users.

RESEARCH BRIEFS

Behavioral inhibition is a strong risk factor for social anxiety disorder

Jacqueline Clauss, & Blackford, JU (2012). Behavioral Inhibition and Risk for Developing Social Anxiety Disorder: A Meta-Analytic Study**,** *Journal of the American Academy of Child & Adolescent Psychiatry* 51(10): 1066-1075.

Social anxiety disorder (SAD)—the persistent and intense fear of being scrutinized by others—is a highly prevalent anxiety disorder that affects approximately 1 in 10 people in their lifetime. SAD is chronic, typically develops in adolescence, and can cause profound functional impairment at school and with peers, eventually resulting in lower educational attainment and wages, and fewer quality relationships during adulthood. SAD also confers risk for development of comorbid psychiatric disorders; 70% of adults with SAD will eventually develop a comorbidity such as major depression or substance abuse. Given the significant disability associated with SAD, early identification and intervention of at-risk adolescents would be ideal. One of the strongest known risk factors is behavioral inhibition (BI)—the chronic tendency to respond to novel people, places, and objects with wariness and avoidance behaviors. BI is heritable, emerges in childhood, and is present in 15-20% of individuals. However, the degree of risk associated with BI varies from study to study, making the size and potential clinical significance of the association unclear. Therefore, Clauss and colleagues conducted a meta-analysis to quantify the association between childhood BI and risk for SAD. The results of this analysis show that BI confers a greater than sevenfold increase in risk of developing SAD (odds ratio = 7.59), meaning that over 40% of children with BI (as opposed to 10% of the general population) will go on to eventually develop SAD. This indicates that BI is a principal and clinically significant predictor of risk for SAD, and an important indicator for early intervention and prevention.

Swimming worms provide insight into new modifiers of dopamine signaling

J Andrew Hardaway, & Hardie, SL, Whitaker, SM, Baas, SR, Zhang, B, Bermingham, DO, Lichtenstein, AJ, & Blakely, RD (2012). Forward Genetic Analysis to Identify Determinants of Dopamine Signaling in Caenorhabditis elegans Using Swimming-Induced Paralysis , *G3 (Bethesda)* 2(8): 961-975.

The dopamine (DA) pathway is complex in its control of both cognitive and motor behavior, with altered signaling resulting in several different brain diseases. Despite the current knowledge on key components of the pathway, investigation into novel modifiers of the pathway may illuminate further mechanisms behind DA-associated brain

diseases. Hardaway and colleagues took advantage of an unbiased, forward genetics approach using the genetically amenable, invertebrate model system, Caenorhabditis elegans (C. elegans). These forward genetic screens were based on a previously established dopamine transporter (DAT, or DAT-1 in worms)-associated phenotype known as Swip, or Swimming-induced paralysis, that requires the function of the DA receptor DOP-3 in worms. Hardaway and colleagues isolated two novel dat-1 alleles (vt21 and vt25 lines) that displayed altered protein expression and trafficking to the cell surface. Interestingly, they also discovered two new lines, vt25 and vt29, that do not contain mutations in dat-1. While vt25 animals did not show a strong swip phenotype but was dependent on DOP-3, vt29 was nearly 100% swip penetrant in a CAT-2 (tyrosine hydroxylase in worms)-dependent manner. Moreover, vt25 and vt29 also showed differential swip rescue responses to DAT overexpression, with both lines showing reduced dopamine content. Interestingly, vt29 showed full resistance to 6-OHDA similar to dat-1 animals, while vt25 only showed partial resistance, indicating that the Swip-responsive gene in this line modulates DAT-1 activity. Therefore, this study further validates the Swip phenotype as an appropriate readout of DAT activity, while also offering novel genomic loci that may provide insight into therapeutics targeting new DA-modulating genes.

Modifier genes in epilepsy show sex-specificity and transcriptome differences

Nicole A Hawkins, & Kearney, JA (2012). Confirmation of an epilepsy modifier locus on mouse chromosome 11 and candidate gene analysis by RNA-Seq , *Genes Brain Behavior* 11(4): 452-460.

About 1% of the worldwide population experiences some form of epilepsy, which is a neurological disorder that often affects voltage-gated sodium channels. Several genes have been linked to the syndrome, particularly mutations in genes associated with voltage-gated sodium channels such as SCN1A and SCN2A. Using a transgenic mouse model ($Scna2^{Q54}$) characterized by slowed channel inactivation and assiduous sodium current, Nicole Hawkins and JA Kearney investigated in their 2012 study how variability in clinical phenotype relates to genetic strain background as additional genes modulate the mutated gene. In fact depending on the genetic strain, $Scna2^{Q54}$ mice show varying degrees of epileptic activity including delayed seizure onsets and improved survivals. Moreover, two loci on chromosome 11 (Moe1) and 19 (Moe2) have been characterized to influence severity of $Scna2^{Q54}$ and fine mapping of Moe2 has lead to Kcnv2 being identified as a candidate gene. In the present study, focus on Moe1 revealed two modifiers, which are sex-specific and include transcriptome differences and the encoding of single-nucleotide polymorphisms. Subsequent analysis of gene function and expression patterns suggested several modifier genes such as voltage-gated calcium channel subunits Cacna 1g and Cacnb 1 as well as a transcription factor Hlf.

Sex-specific differences in the biologic mechanism of posttraumatic stress disorder

Asante Kamkwalala, Norrholm, SD, Poole, JM, Brown, A, Donley, S, Duncan, E, Bradley, B, Ressler, KJ, & Jovanovic, T (2012). Dark-enhanced startle responses and heart rate variability in a traumatized civilian sample: putative sex-specific correlates of posttraumatic stress disorder**,** *Psychosomatic Medicine* 74(2): 153-159.

Prior research has linked trauma with an increased risk for posttraumatic stress disorder (PTSD). In a study in 2012, Asante Kamkwalala and colleagues sought out to investigate characteristics of the biologic mechanisms of PTSD. They specifically looked at the dark-enhanced startle response (indicator for anxiety) and measures of heart rate variability and found that both measures may be sex-specific. They recruited 14 highly traumatized adults and recorded psychophysical responses of the eye bling reflex and heart rate variability during a startle paradigm with light and dark phases. Their data shows that female patients exhibited higher startle response magnitudes. Although there appeared to be no sex-difference in heart rate variability, male patients had increased high frequency heart rate variability compared to male controls. Kamkwalala and colleagues suggest that the observed sex-differences may be associated with the limbic system, specifically with the sexually dimorphic bed nucleus of the stria terminalis, which is about 2.5 times larger in men.

CaMKII inhibition regulates medium spiny neuron activity

Jason R Klug, Mathur, BN, Kash, TL, Wang, HD, Matthews, RT, Robison, AJ, Anderson, ME, Deutch, AY, Lovinger, DM, Colbran, RJ, & Winder, DG (2012). Genetic inhibition of CaM-

KII in dorsal striatal medium spiny neurons reduces functional excitatory synapses and enhances intrinsic excitability, *PLoS ONE* 7(9): e45323.

CaMKII is a kinase important for both the presynaptic and postsynaptic neuron functions. Though its role in learning and memory are well known, a more unexplored territory is the role of CaMKII in the striatum, where it is highly expressed in GABAergic medium spiny neurons (MSNs). The striatum is important in the pathophysiology of two dopamine-related diseases: Parkinson's disease (PD) and addiction. Since dopaminergic neurons modulate the firing of striatal MSNs, identifying the factors that regulate MSN firing behavior is important to understanding disease mechanisms. In Klug et al. (2012), the Winder lab explores the role of CaMKII in striatal MSNs using a transgenic mouse model that expresses an EGFP-linked CaMKII inhibitor peptide (EAC3I) in MSNs.

Inhibiting CaMKII resulted in a reduction in activity-dependent excitatory postsynaptic currents (mEPSCs), which was attributable to reduced synapse number. CaMKII promotes membrane trafficking of the AMPA receptor GluA1 subunit, which is important for activity-dependent synapse potentiation. MSNs from GluA1 knockout mice displayed similar problems with firing frequency. Additionally, CaMKII inhibition resulted in increased resting membrane potential and input resistance, markers of the cell's intrinsic excitability.

The alterations in MSN firing behavior and membrane potential, and possible differences in AMPA receptor trafficking that come with CaMKII inhibition, demonstrate that CaMKII is important for the membrane properties that allow MSNs to fire and propagate signals. In disorders such as PD and addiction, the firing of dopaminergic neurons in the striatum can be severely depleted, thereby altering the behavior of postsynaptic MSNs. Previous work has shown that CaMKII inhibition ameliorates disease symptoms in animal models of PD and addiction. A possible mechanism could be that the upregulation of MSN firing after CaMKII inhibition compensates for reduced input from dopaminergic neurons, indicating that CaMKII inhibition could someday serve as a therapy for PD and addiction.

A precise measure of manganese levels allows more accurate detection of modulators

Kevin K Kumar, Aboud, AA, Patel, DK, Aschner, M, & Bowman AB (2013). Optimization of Fluorescence Assay of Cellular Manganese Status for High Throughput Screening, *Journal of Biochemical and Molecular Toxicology* 27(1): 42-49.

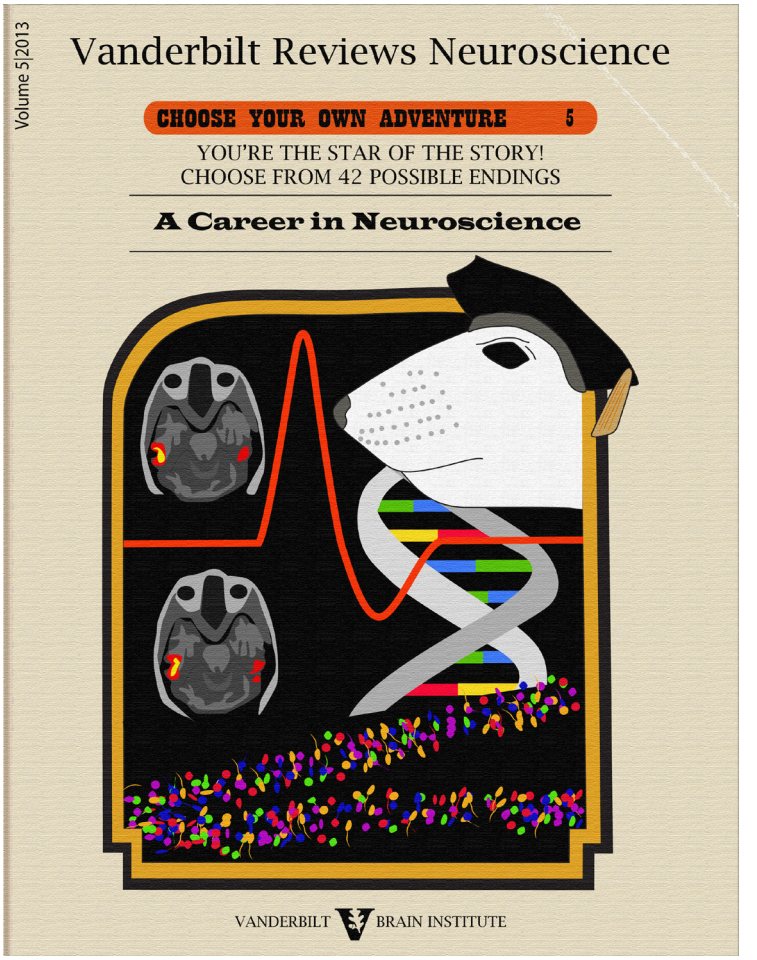
The Bowman Lab has previously developed the Cellular Fura-2 Manganese Extraction Assay (CFMEA) for quantifying intracellular manganese (Mn) levels. In his article in the Journal of Biochemical and Molecular Toxicity, Kevin Kumar and colleagues explain how he has optimized this assay for high throughput screening (HTS) by adjusting concentrations and analysis tools for efficiency in identifying modulators with increasing signal-to-background ratios. In addition, Kumar and colleagues identified and decreased variability among samples, achieving a Z-factor required for HTS.

Mn neurotoxicity causes basal ganglia dysfunction and neurodegeneration. In addition, Mn exposure is an environmental risk factor for Parkinson's disease (PD), and modulation of neuronal Mn status has been observed in the pathogenesis of Huntington's disease (HD). Identification of modulators of Mn accumulation in neurons is a potential route to identify novel treatments for these debilitating neurodegenerative disorders. The authors used an immortalized murine striatal cell line exposed with physiologically relevant Mn concentrations. The first steps was to determine a cell density (cells/well) and exposure paradigm that would yield 50% quenching of the vehicle fura-2 signal. This was found to be 10,000 cells/well exposed for 60 minutes. Subsequently, the authors identified that variance was least in a fura-2 concentration of 0.75 μM and that precision was highest in an extraction volume of 125 μL. Finally, analyzing normalized fluorescent levels, rather than raw levels, decreased batch-to-batch variability. With these new parameters, CFMEA for HTS reliably detects cellular Mn levels. CFMEA is currently being utilized for HTS of small molecule libraries for modulators of manganese accumulation and toxicity.

On the Cover

A career in neuroscience is multi-faceted. This year's cover art was specifically chosen to represent all the wonderful career directions the field of neuroscience in general and our program in particular offers. Eva Sawyer graciously agreed to design a cover inspired by the *Choose Your Own Adventure* book series, as our students and faculty choose their own adventure, too.

Eva is a fourth year graduate student in Dr. Ken Catania's laboratory, where her research meets her interest in how evolution and ecology shape sensory systems. In Dr. Catania's lab, she studies the somatosensory system in the star-nosed mole, a small subterranean animal with an elaborate somatosensory organ on its nose. The star-nosed mole has 22 rays on its nose that it uses to navigate its world. Eva's specific focus is on the ray representation along the somatosensory pathway and the preserved somatosensory map with emphasis on subcortical somatosensory nuclei such as the trigeminal brainstem complex and the ventral posterior medial nucleus of the thalamus. Eva investigates the ray-map using both neuroanatomical techniques such as specific immunohistochemical stains as well as neurophysiological techniques looking at multi-unit extracellular neuronal recordings during somatosensory stimulation of the mole rays. Eva also recently received a Ruth L. Kirschstein National Research Service Award to support her research endeavors.



CANDIDATE
REVIEWS

Regulation of the Dopamine Transporter Through Trafficking-Dependent and -Independent Mechanisms

Daniel Bermingham

The dopamine transporter (DAT) is a vital protein involved in maintaining dopamine (DA) homeostasis in the brain by mediating reuptake of synaptic DA back into the presynaptic termi-nal. The regulation of this protein is critical for ensuring proper dopaminergic signaling, and its dysregulation can have dire consequences for a number of behaviors and neurological pro-cesses that are modulated by DA signaling. DAT activity can be regulated in both a positive and negative manner; the mechanisms of regulation involve both trafficking of the protein to and from the surface, as well as modulation of intrinsic transport activity independent of trafficking. Discussion of these trafficking-dependent and -independent modes of regulation will be the focus of this review.

Keywords: *Dopamine, transporter, presynaptic, trafficking, monoamines*

Introduction

The neurotransmitter dopamine (DA) is important for modulating many aspects of physiology and behavior, including motivation[1], movement[2], reward[3] and attention[4]. Importantly, perturbations to the central dopaminergic systems are associated with many disease states such as Parkinson's disease[2], attention deficit/hyperactivity disorder (ADHD)[5], schizophrenia[2] and addiction[6]. Therefore, understanding regulation of dopaminergic signaling is vital for understanding the pathophysiology of these diseases, as well as for gaining insights into the behaviors modulated by this neurotransmitter.

Presynaptic regulation of DA signaling is a dynamic process that can occur at many levels. These include regulation of DA synthesis by the enzyme tyrosine hydroxylase[7], excitability of the presynaptic neuron[8],and, most importantly for this review, regulation of the activity of the dopamine transporter (DAT), which acts to reuptake synaptic DA back into the presynaptic terminal to halt its signaling[9]. DAT is a twelve transmembrane domain protein that belongs to the sodium- and chloride-dependent (SLC6) family of transporters, of which the serotonin, norepinephrine, and GABA transporters are also members[10]. This family of transporters couple movement of their substrates across membranes to movement of sodium and chloride ions down their electrochemical gradients, allowing for energy-independent movement of substrate across the membrane[10]. DAT itself is the target for therapeutic agents such as the ADHD medications methylphenidate (Ritalin) and amphetamine formulations (e.g., Adderall)[11], and variants in this protein have been found to be associated with ADHD[12-15]. This, along with the fact that DAT is the target of addictive psychostimulants such as cocaine[9], amphetamine[11], and methamphetamine[16], implicates the importance of this protein for the pathophysiology of many of the disease states associated with altered DA signaling.

Importantly, DAT is not a static protein, and tight regulation of its activity has been observed experimentally[17-20]. Much research has demonstrated trafficking-dependent regulation of DAT through movement of the protein to and away from the surface by a number of interacting proteins and post-translational modifications, many of which will be discussed below. However, trafficking-independent regulation, where the intrinsic activity of DAT is modulated without trafficking to other compartments, has not been described as extensively. This review will focus on discussing these two broad classes of regulation that likely work in concert to achieve tight regulation of the activity of this vital protein.

Trafficking-dependent downregulation of DAT

The most robustly observed mechanism of DAT regulation,

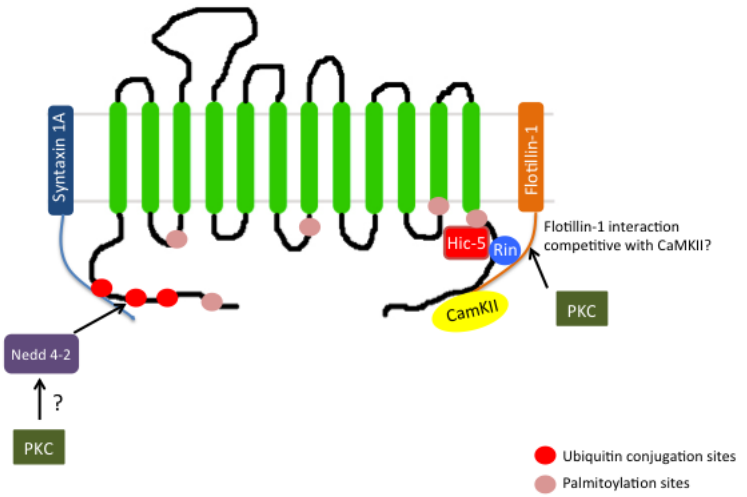


Figure 1: *Modulation of DAT activity.* DAT activity can be modulated by a number of interacting proteins, though which of these proteins may act together, or which may be exclusive and/or competitive is not entirely clear. Importantly, the site of interaction of Flotillin-1 has not been mapped, but has been placed based on evidence of potential competition with CaMKII. Post-translational modifications such as palmitoylation and ubiquitination also play important roles in regulating DAT activity and surface expression. Direct phosphorylation of the transporter has not been shown to regulate activity per se, but is important for regulating efflux-competent states of DAT.

downregulation of transport through trafficking of the transporter, is achieved through a large network of interacting proteins, the list of which is ever-expanding. Downregulation of DAT by Protein Kinase C (PKC) activation is among the most frequently studied mechanisms of DAT regulation, and is mediated by a number of proteins[21-25]. Activation of PKC by phorbol esters such as PMA[23, 24, 26] or by activation of G_q-coupled G-protein coupled receptors[27] induces a rapid reduction in surface DAT and a concomitant increase in intracellular DAT[28]. This redistribution of DAT is mediated by a clathrin-dependent mechanism, as knockdown of either clathrin heavy chain or dynamin II inhibits both constitutive as well as PKC-triggered endocytosis[26, 29]. However, direct phosphorylation of DAT by PKC is not required for internalization of the protein, suggesting that potentially another DAT-interacting protein is phosphorylated either by PKC or a downstream kinase to mediate this effect[30]. It was recently reported by Cremona et al. that the membrane raft-associated protein Flotillin-1 was required for internalization of DAT upon PKC activation, and that phosphorylation of this protein at Ser315 is also required for this effect[31]. These results suggest that Flotillin-1 may be either a direct or indirect target for phosphorylation by the PKC signaling cascade. It was also observed that Flotillin-1 was required for maintaining membrane raft-association of DAT, potentially pointing toward the importance of this localization for PKC-regulated endocytosis.

Further evidence of the importance of membrane microdomain targeting in PKC-regulated endocytosis has recently been reported. An ADHD-associated variant in the dopamine transporter (R615C) was shown to constitutively internalize and recycle back to the membrane at an accelerated rate, and was insensitive to PKC- and amphetamine (AMPH)-stimulated endocytosis[12]. Importantly, this variant showed reduced association with Flotillin-1 and reduced membrane raft localization, as measured by colocalization with an Alexa 647-conjugated cholera toxin B, which marks membrane raft-enriched GM1 Ganglioside. This reduction in association with Flotillin-1 and membrane rafts is contrasted by a significant increase in association with Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). It is possible that the reduced association between the 615C variant and Flotillin-1 may be due to the fact that CamKII and Flotillin-1 interactions with DAT are competitive due to overlapping sites of interaction. Therefore, an increased affinity for CaMKII might prevent Flotillin-1 interactions and raft association, though this is just speculation at this point since the site of interaction between Flotillin-1 and DAT has not been mapped (**Figure 1**). Regardless, these altered associations in this PKC- and AMPH-induced trafficking-insensitive DAT variant certainly provide further support for the importance of the Flotillin-1 interaction and raft localization for

Membrane rafts:
Cholesterol-rich microdomains that are thought to organize signaling molecules into discrete regions of the plasma membrane.

Clathrin:
A protein that mediates endocytosis of vesicles, a process that is important for many cellular physiological processes, including internalization of cell-surface proteins.

PKC and AMPH-triggered endocytosis of the transporter.

Another recent study identified another DAT-interacting protein, the plasma membrane-associated GTPase, Rin, which is involved in mediating the effects of PKC on DAT[32]. Utilizing yeast two-hybrid, GST pull down, and FRET approaches, it was demonstrated that Rin directly associates with residues 587-596 of the DAT C-terminus, and that expression and activity of Rin is required for PKC-triggered endocytosis of the transporter. It was also shown that Rin and DAT interactions increase with PKC activation. Furthermore, Rin/DAT interactions were shown to occur preferentially in membrane rafts, again pointing toward the importance of this localization for PKC-regulated DAT trafficking. Unfortunately, attempts at demonstrating an interaction *in vivo* in dopaminergic neurons were unsuccessful due to technical issues, so the relevance of this interaction is unknown. Importantly, Rin is present in striatal tissue and a number of DAergic cell lines, supporting a potential role in endogenous DAT regulation. The role that Rin plays in DAT regulation is unclear, though it is possible that it helps regulate movement of DAT into or out of membrane raft microdomains, a process that has been suggested to be important for PKC regulation of DAT. However, more work is necessary to clarify just what role this interaction plays in DAT trafficking by PKC-dependent pathways.

It has been also been shown that PKC activation results in ubiquitination of DAT, and that three lysine residues in the N-terminus of DAT that act as ubiquitin conjugation sites are required for PKC-dependent endocytosis of the transporter[33]. Utilizing a large-scale RNAi screen for genes involved in PKC-dependent endocytosis, Sorkina et al. identified the E3 ubiquitin ligase, Nedd4-2, as being necessary for this process, as loss of this protein by siRNA-mediated knockdown abolished DAT ubiquitination[34, 35]. These data suggest that PKC activation induces ubiquitination of DAT by Nedd4-2, and that this process is necessary for PKC-regulated endocytosis of the transporter. It is possible that PKC itself regulates Nedd4-2 activity or recruitment to DAT, though this has not been shown experimentally. Also, the interplay between this Nedd4-2 dependent mechanism and the interaction with Flotillin-1 or Rin has yet to be investigated.

Another interesting DAT-interacting protein that appears to be involved in trafficking of the transporter is the focal adhesion protein Hic-5. This interaction was initially identified by the yeast two-hybrid system, and verified by co-immunoprecipitation from striatal extracts and immunostaining that showed presynaptic colocalization of these two proteins[36]. Co-expression of DAT and Hic-5 in HEK293 cells resulted in a decrease in transport and surface expression of DAT compared to expression of DAT alone. These results suggest that the association between DAT and Hic-5 may be important for surface trafficking of DAT. In further support of this, work done in platelets by Carneiro and Blakely has shown that Hic-5 also associates with the serotonin transporter (SERT)[37]. Interestingly, treatment with PMA, which also induces internalization of SERT, increases association between SERT and Hic-5 at times that correlate with decreases in SERT surface expression. If a similar mechanism occurs with Hic-5 and DAT, it may be the case that Hic-5 is involved in the network of proteins mediating PKC-regulated DAT endocytosis, though direct evidence for this has yet to be presented. Also worth noting is the fact that this interaction between Hic-5 and DAT occurs in the membrane-proximal region of the C-terminus DAT, very close to the interaction site of Rin. It is possible that these proteins function together to regulate DAT trafficking, or may be competitive for binding to DAT and may mediate different forms of DAT regulation, though these possibilities have yet to be investigated.

Importantly, it should be noted that the timing and cellular contexts of these various PKC-related mechanisms of DAT endocytosis remain unclear. For instance, it is unknown if these proteins work in tandem in DAergic terminals through a single PKC-related mechanism of DAT endocytosis, or if there is exclusivity between these different interactors depending on factors such as the active signaling pathways or the membrane microdomain localization of DAT. Foster et al. demonstrated that treatment with β–PMA causes PKC-α to be recruited to membrane rafts and may preferentially regulate endocytosis of DAT in these fractions through some of the mechanisms mentioned above. However, it is possible that activation of other PKC isoforms by other signaling pathways may lead to modulation of DAT endocytosis by entirely separate mechanisms. Sakrikar et al. have proposed a model based on work with the R615C DAT variant that postulates that DAT endocytosis occurs in both regulated and constitutive manners that depend upon microdomain localization. In this model, PKC regulates endocytosis from membrane rafts, whereas DAT localized outside of these rafts endocytoses in a constitutive manner. This is consistent with the finding that PKC-α moves into raft fractions after PMA treatment, but Foster et al. also showed that PKC activation does not

reduce surface DAT in raft fractions. These inconsistencies may reflect the different cell types used or the methods employed, or it may be the case that PKC-α mediates a trafficking-independent mode of DAT regulation, and other isoforms, such as PKC-β underlie mechanisms of trafficking-dependent regulation of DAT. Clearly, the picture of PKC regulation of DAT is quite complicated at this point, and this complication is made worse by the inconsistency in cell lines and techniques employed by various groups. Regardless, it certainly seems as if PKC regulation of DAT surface expression may actually occur through a number of mechanisms; future work on the interaction of these various PKC-related mechanisms, as well as the PKC subtypes mediating these effects, may clarify where and when these different pathways may modulate DAT surface expression *in vivo*.

The neuronal SNARE protein Syntaxin 1A is another DAT regulatory partner whose interaction appears to have a number of functional consequences for DAT activity. Tissue treatment with Botulinum Neurotoxin C (BoNT/C) results in degradation of Syntaxin 1A, and treatment of rat striatal tissue with this toxin causes an increase in DA uptake and DAT surface levels[38]. Conversely, heterologous co-expression of these two proteins results in decreased DA uptake and DAT surface levels compared to DAT expression alone. Together, these results suggest that the interaction between these two proteins promotes suppression of DA uptake through reduced DAT surface levels. Importantly, PMA-induced endocytosis of DAT was intact even with BoNT/C treatment, suggesting that the Syntaxin 1A/DAT interaction is not required for PKC-triggered endocytosis of the transporter.. This interaction is interesting because it may have relevance for the localization of DAT to sites of release, as Syntaxin 1A is an important member of the SNARE complex that mediates vesicular fusion and neurotransmitter release. What the relevance of this interaction is remains unclear, but it is important to note that Syntaxin 1A is also essential for facilitating an efflux-competent state of DAT and decreases DAT channel currents, which, combined with the apparent effects on trafficking, likely have important functional consequences for how DAT behaves endogenously.

SNARE:
A family of proteins that mediate docking and fusion of vesicles to the plasma membrane, which allows for the release of neurotransmitter.

Trafficking-dependent upregulation of DAT

There are a number of signaling pathways that have been shown to promote surface expression of DAT through trafficking-dependent mechanisms. For instance, insulin increases dopamine uptake by increasing surface expression of DAT, and this increase can be blocked by inhibition of Phosphatidylinositide 3-kinase (PI3K)[39]. Importantly, PI3K inhibition also reduces basal uptake and surface expression in a dynamin-dependent manner, suggesting that this regulation is vital for opposing endocytosis of the protein by other pathways such as PKC, etc[39]. Also vital for this process is the kinase Akt, whose level of phosphorylation increases upon insulin stimulation[40]. Constitutively active Akt prevents AMPH-induced endocytosis of DAT, and inhibition of Akt reduces DAT uptake and surface expression, supporting its role in the insulin/PI3K pathway that promotes surface expression of DAT.

DAT activity can also be modulated by interactions with the D2 dopamine receptor (D2R). D2Rs are expressed both postsynaptically and presynaptically, and can be alternatively spliced into short and long isoforms (D2S and D2L), with the D2S isoform as the predominant presynaptic isoform. It has been observed that D2-deficient animals have reduced DAT function[41], and heterologous expression of D2 and DAT suggests that this D2 effect on DAT occurs cell-autonomously[42]. By co-expressing D2 and DAT, it has been shown that these proteins directly interact[43, 44], and increases uptake and DAT surface expression in heterologous systems compared to singly transfected DAT[43]. In addition to this direct interaction, there also appears to be a functional interaction between these two proteins. When DAT and D2S were co-expressed in cells, the D2 agonist quinpirole significantly increased dopamine

uptake and DAT surface expression, and a specific ERK1/2 antagonist could block this increase[44]. This suggests that D2 modulation of DAT activity likely occurs via at least two mechanisms: a direct interaction of the proteins promoting increased surface expression of the transporter, as well as a functional interaction that involves D2-dependent ERK1/2 signaling cascades.

An important thing to keep in mind when considering how these signaling pathways influence DAT regulation is that these pathways overlap and interact in many ways. For instance, in addition to activating PI 3-kinase, insulin can also activate PKC signaling pathways via PLC gamma, which would presumably oppose PI 3-kinase upregulation of DAT. It is likely that compartmentalization of DAT with its regulatory partners helps organize these regulatory mechanisms in manners that aren't well understood, and it should always be kept in mind that much of the work on DAT regulation is done in heterologous expression systems that may not accurately reflect the environment in which DAT is endogenously expressed. Because of this, care must be taken in how the results of such studies are interpreted until they are repeated in endogenous DAT-expressing systems.

Trafficking-independent downregulation of DAT

In recent years, it has become clear that trafficking of DAT alone cannot explain regulation of its activity, and that there are trafficking-independent modes of regulation of this protein. Foster et al. initially reported findings that suggest that reduction in DAT activity by PMA-induced PKC activation could only partially be explained by internalization of the transporter[45]. They demonstrated this by inhibiting clathrin-mediated endocytosis using either the chemical inhibitor Concanavalin A (Con A) or a dominant-negative dynamin. This inhibition was sufficient to prevent internalization of the protein, but only partially prevented PKC-induced downregulation of transport activity. Also, using a cholesterol depletor, methyl-β–cyclodextrin (MβC), it was demonstrated that PKC-induced downregulation was also partially inhibited by loss of cholesterol, but DAT internalization was about equivalent to PMA treatment alone, suggesting a loss of PKC-induced downregulation that was independent of trafficking. In further support of this, another study showed that PKC-induced downregulation of DAT in synaptosomes occurs even in the presence of high sucrose, which blocks endocytosis[46]. These lines of evidence suggest that PKC causes a decrease in DAT activity via a mechanism that does not require internalization of the protein.

The association of DAT with cholesterol-rich membrane rafts has been well characterized, and this association can be decreased by MβC treatment and increased by treatment with water-soluble cholesterol (wsChol). By augmenting membrane cholesterol of DAT-transfected HEK cells. as well as striatal synaptosomes with wsChol, Hong and Amara demonstrated that binding of cocaine-analogs [^{125}I] RTI-55 and [^{3}H]WIN35428 was significantly increased compared to untreated controls[47]. They also showed that binding of Maleimide-PEO_2-biotin to DAT, which specifically recognizes sulfhydryl (–SH) moieties on surface-accessible cysteine residues, is increased with no change in total surface DAT. Using site-directed mutagenesis, the site of increased reactivity was found to be cysteine 306, and its increased availability to the –SH-specific biotin was attributed to an increase in outward conformation of the DAT protein. This cholesterol-dependent change in DAT conformation may underlie some of the modulation of DAT activity by altered membrane cholesterol content, and may represent a mechanism through which altered cholesterol-rich membrane raft association of DAT can regulate transport, independent of trafficking to and away from the cell surface.

Trafficking-independent upregulation of DAT

Though very little direct evidence for trafficking-independent upregulation of DAT activity has been observed, there are a few findings that indicate that this may occur. Foster and Vaughn have shown that DAT is palmitoylated, and that this modification has functional consequences for transport[46]. By inhibiting palmitoyl acyltransferase using 2-bromopalmitate (2BP), which prevents protein palmitoylation, they showed that blocking palmitoylation of DAT induces a rapid reduction in transport with no changes in DAT protein or surface levels at early time points. It is important to note that 2BP inhibited palmitoylation of DAT by about 40% within 45 minutes, suggesting that palmitate turnover is quite rapid, and that this modification may be used to acutely regulate DAT activity. If this is the case, then palmitoylation and depalmitoylation may represent mechanisms by which DAT kinetics can be rapidly up- or downregulated, independent of trafficking of the protein. Additionally, since palmitoyl groups can mediate interactions between proteins and membrane lipids, the palmitoylation status of DAT may impact its membrane microdomain localization and, therefore, its regulation, providing a potential mechanism through which activity

and surface expression may be altered by this modification.

Further evidence from the related transporters SERT, as well as the norepinephrine transporter (NET), demonstrates that trafficking-independent upregulation of transporters does occur, and it is tempting to think that this mode of regulation may generalize to other family members such as DAT. For instance, activation of adenosine A3 receptors on serotonergic neurons increases SERT activity by a mechanism that is partially independent of trafficking to the surface[48-54]. The signaling pathways involved have been worked out, and it appears that activation of PKG and p38 MAP kinase underlies this upregulation[50-53]. In the case of NET, it has been demonstrated that insulin stimulation also activates p38 MAP kinase, and this activation induces upregulation of transport without any significant increase in surface expression[55, 56]. This raises the question of whether mechanisms that upregulate DAT activity may be doing so via similar mechanisms that may be due in part to trafficking-independent activation. It seems reasonable to think that perhaps trafficking-independent upregulation of DAT has not been reported because of a lack of temporal resolution in monitoring DAT surface expression. For example, many studies that showed increased uptake and a concomitant increase in DAT surface levels only looked at DAT surface levels at later time points, and may have missed an earlier increase in DAT activity prior to trafficking of DAT to the surface. Hopefully, future work will begin to clarify whether this mode of regulation is indeed employed to regulate DAT, as has been shown for related transporters.

Conclusions

Understanding how DA homeostasis is maintained and dynamically regulated is essential for gaining insights into how DA mediates its effects on behavior, and how dysfunction of this system can lead to diseases such as ADHD, schizophrenia, and addiction. At the center of DA signaling regulation is DAT, whose activity is vital for controlling the proper amount of synaptic DA during neurotransmission. Because DAT dysfunction has been shown to be associated with a subset of individuals with diseases such as ADHD, it is imperative that there be a focus on studying how this protein is regulated in order to understand its role in these disease states, as well as in normal cognition and behavior. As this review has shown, there are many different modes of DAT regulation involving trafficking and modulation of intrinsic transport activity, though when and where these pathways may regulate DAT *in vivo* and how they may interact with one another remains poorly understood. As these regulatory networks of proteins are worked out further, it will hopefully expand out understanding of DA signaling in the brain and open up avenues for treating individuals with DA-related brain disorders.

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CANDIDATE
REVIEWS

The Waiting Game: How Studying Anticipatory Processing Can Provide New Insight into the Neural Circuit of Dysfunction in Anxiety Disorders

Jacqueline Clauss

Anticipation of upcoming aversive or threatening events is a highly adaptive psychological process. During anticipation, individuals consider the current environment and prior memories to initiate cognitive and motor processes. Anticipation of aversive stimuli has been studied in a number of neuroimaging paradigms, and results have shown that anticipation results in increased activation in a network of brain regions. This network includes emotion reactivity regions—the amygdala and insula, which signal threat and arousal—and emotion regulation regions—the dorsal anterior cingulate cortex and dorsolateral prefrontal cortex, which signal action and cognitive control. Balance between emotion reactivity regions and emotion regulation regions is necessary for the successful preparation for an upcoming aversive event. Anticipation can also be maladaptive, such as in anxiety disorders, and understanding anticipatory anxiety can provide new insight into dysfunction in the neural circuit in anxiety disorders. Patients with anxiety disorders may engage anticipatory processes to neutral or mildly aversive stimuli. When patients with anxiety disorders anticipate aversive stimuli, they have greater activation of emotion reactivity regions and less activity of emotion regulation regions, relative to controls. Among patients with anxiety disorders, patients who have greater activation of emotion regulation regions typically experience fewer symptoms. Individuals with social phobia typically experience extreme anticipatory anxiety prior to social situations; anticipatory anxiety has been studied using speech anticipation paradigms. In patients with social phobia, relative to controls, anticipation of social stimuli is associated with increased activity of emotion reactivity regions, and less activity of emotion regulation regions in patients with social phobia, relative to controls. Studying anticipation of social stimuli in patients with social phobia may lead to greater understanding of the pathophysiology of social phobia and help to identify new targets for treatment and prevention.

Keywords: *Anxiety, anticipation, fear, amygdala, dorsal anterior cingulate, dorsolateral prefrontal cortex, insula, social phobia*

Anticipation of Aversive Events

An upcoming aversive event, such as a having a thesis committee meeting, giving a scientific talk, or taking a test triggers anticipation of that event. Anticipation is often adaptive; during the anticipation of an event, mental and physical preparation takes place. Anticipation of aversive stimuli promotes survival in a changing environment[1]. Anticipation of aversive events can be broken down into a number of overlapping processes (see **Figure 1**): A.) orienting and threat detection;[2] B.) memory recall and evaluation;[3] and C.) motor preparation and cognitive control[4]. During anticipation of emotional stimuli, physiologic reactivity is heightened, suggesting emotional arousal[5,6]. Anticipatory processes may modify behavior, and avoid, prepare for, or alter the aversive event. Cognitive processes, such as reappraisal, distraction, or emotion suppression, may also reduce anxiety and allow for mental preparation for the aversive event. Better understanding of the neural processes of anticipation of aversive stimuli may provide insight into these adaptive responses

Anticipation of aversive events is mediated by emotion reactivity and emotion regulation brain regions. During anticipation of aversive events, common emotion reactivity regions activated include the amygdala[2,4,7–11] and the insula[2,4,8–17], and common emotion regulation regions activated include the dorsolateral prefrontal cortex (dlPFC)[1,4,9,10,13,14,17] and the dorsal anterior cingulate cortex (dACC)[1,2,4,11–13,15,16] (see **Figure 2**). The amygdala detects and attends to aver-

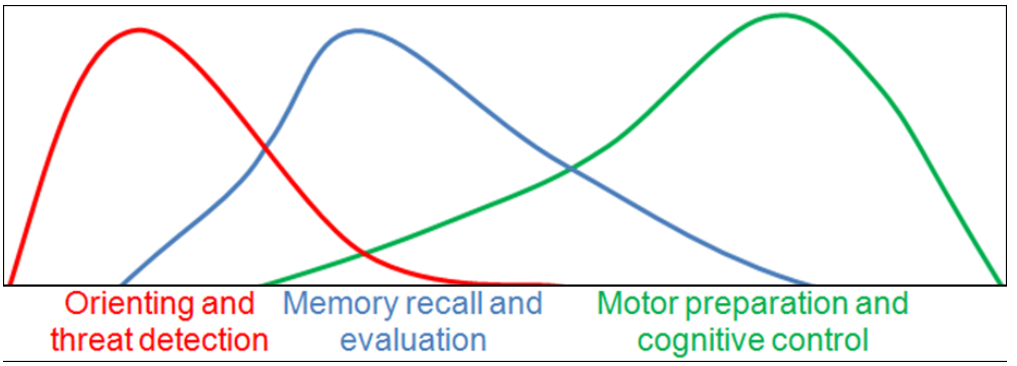

Figure 1: *Phases of anticipatory processing.* Anticipation of aversive events involves several distinct, yet overlapping processes, including orienting and threat detection, memory recall and evaluation, and motor preparation and cognitive control.

sive stimuli,[18,19] activates fear responses,[18] and engages emotional memory[20]. The insula detects and responds to emotional, interoceptive, and autonomic responses,[21] and may represent internal feelings of anticipatory anxiety[9], and amygdala-insula structural connections[22] may play an important role in anticipatory processing. During anticipation of aversive stimuli, emotion regulation regions have a dual role – initiating behavioral and cognitive responses and regulating activation in emotion reactivity regions. The dACC integrates cognitive, motor, and affective processes, and plans future behavioral or emotional responses.[21,23] During anticipation, the dACC may be particularly important in integrating emotional responses and cognitive processes.[21] The dlPFC also engages emotional working memory[24] and exerts cognitive control over emotional responses[25,26]. The dACC and dlPFC are reciprocally connected,[27,28] and dACC projections to the amygdala[29–31] and insula[32,33] may regulate emotion reactivity.

During anticipation of aversive events, cognitive control processes may regulate emotional responses. Cognitive control processes may be implicit (engaged spontaneously) or explicit (instructed); common cognitive control processes include reappraisal, reality checking, and even the use of a placebo, in studies examining response to pain. Cognitive control processes commonly activate the prefrontal cortex, including the dACC and dlPFC[34,35] and suppress activation in emotion reactivity regions, including the amygdala and insula[36]. More frequent use of cognitive reappraisal strategies is related to larger dACC volume[37]. Placebo analgesics are associated with increased activation in the dlPFC and reduced activation in the insula; the magnitude of the placebo effect correlates with the degree of change of neural response in the dlPFC and insula.[36] Individuals who use reappraisal more often during daily life have less amygdala activity during anticipation of aversive stimuli,[25] suggesting that individuals who are more practiced at reappraisal may more effectively suppress emotion reactivity responses. Importantly, during anticipation of negative stimuli, functional connectivity between dACC and left amygdala is increased,[28,30] suggesting that anticipation is a key time for emotion regulation and preparation. Cognitive control strategies may regulate emotional responses[38] and may be adaptive during anticipation of aversive stimuli.

Anticipation of Aversive Events in Anxiety Disorders

In patients with anxiety disorders, anticipation can be maladaptive. Patients often have heightened feelings of anticipation, which can lead to fear, helplessness, and feelings of uncontrollable future threat.[39] Heightened anticipation of aversive stimuli may result in symptoms of excessive worry and avoidance of otherwise safe situations. Greater anticipatory anxiety, and not severity or frequency of symptoms,[40] is related to more avoidance behavior, one of the most detrimental features of anxiety disorders[41]. Patients with anxiety disorders may also display inappropriate anticipation, resulting in anticipation of both phobic stimuli and of relatively minor events[24,42,43]. Patients with anxiety disorders have increased attention bias to threat[44] and are more likely to interpret neutral events as negative[45], both of which may result in increased anticipation. Anxiety disorders are also characterized by ineffective emotion regulation,[46] which may result in withdrawal or avoidance behaviors, rather than the more adaptive anticipation - cognitive control and approach behaviors. Differences in anticipatory processing are likely related to two mechanisms – increased threat detection and less effective emotion regulation.

In support of these hypotheses, patients with anxiety disorders also have increased amygdala[11,24,43] and insula[42,43,47,48] activation during anticipation of aversive stimuli. Most studies have also found less activation of emotion regulation regions during anticipation, including the dorsal anterior cingulate cortex (dACC)[43] and dorsolateral prefrontal cortex (dlPFC)[43,47,48] activation; however, results are not

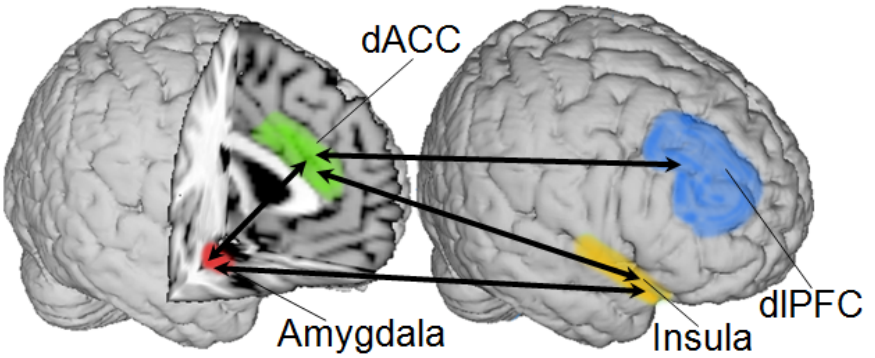

Figure 2: *Neural network of anticipation.* Regions of the brain activated during anticipation of aversive stimuli include the amygdala, insula, dorsal anterior cingulate cortex (dACC), and dorsolateral prefrontal cortex (dlPFC). Connectivity between emotion reactivity regions (amygdala and insula) and emotion regulation regions (dlPFC and dACC) has been observed.

consistent, and a few studies have found increased dACC[42] and dlPFC[24] activation during anticipation of aversive stimuli. Prefrontal cortex regions that are close in anatomic position, may have distinct functions and engage separate processes,[49] and our understanding of the function of prefrontal cortex regions is evolving over time (for example, see [50] and [51]). Further study is needed to determine the precise location and function of changes in prefrontal cortex activity in anxiety disorders. Unlike healthy controls,[25] when patients with social phobia engage cognitive control mechanisms during anticipation of aversive stimuli, they do not show increased activation of prefrontal cortex regulation regions[52].

Patients with anxiety disorders who are better able to engage emotion regulation regions during anticipation of aversive stimuli may have higher functioning, fewer symptoms, and better response to treatment. For example, greater dlPFC activation during anticipation of negative images is associated with fewer PTSD symptoms and better executive functioning.[47] In patients with generalized anxiety disorder, greater dACC activation during anticipation, was associated with greater reduction in anxiety and depression symptoms following treatment.[11] In summary, in patients with anxiety, greater activity of emotion regulation regions during anticipation of aversive events is associated with higher functioning and better treatment response.

Individuals with high trait anxiety are at high risk for developing anxiety disorders, and have increased attention bias to threat[53]. High trait anxious individuals show similar patterns of anticipation of aversive stimuli, including increased activation of the amygdala,[54] insula,[14,48] and dlPFC[14]. Degree of trait anxiety was positively associated with activation in the amygdala and insula during anticipation of aversive stimuli,[9] and high trait anxiety is associated with increased connectivity between the insula and emotion regulation regions, including the dlPFC and dACC.[14] In individuals with high trait anxiety, greater activation of emotion regulation regions and greater coupling between emotion regulation regions and emotion reactivity regions may compensate for heightened emotion reactivity in anxiety. Greater activation of emotion regulation regions during anticipation may protect high trait anxious people from engaging in withdraw or avoidance behavior, and may "protect" against development of anxiety disorders; however, this has not been explicitly tested.

Focus on Anticipation of Aversive Social Stimuli in Social Phobia

Social phobia is defined as the fear or avoidance of one or more performance or social evaluative situations, especially those in which an individual is exposed to social evaluation or scrutiny by others. Symptoms of social phobia are often most prominent during the anticipation of social situations and is accompanied by heightened physiological arousal,[24,55] anticipatory anxiety,[24,55] and negative self-beliefs[56,57]. Understanding the neurobiology of anticipation of social situations has the potential to advance our understanding of the pathophysiology of social phobia.

Anticipation of aversive social stimuli in social phobia has typically been measured using a speech anticipation task, which reflects some of the common triggers of social phobia – fear of social evaluation. Subjects are given several minutes to prepare a speech on a given topic, and then they deliver the speech to a group of experimenters or in front of a video camera. Anticipation of public speaking increases state anxiety,[24,55] negative affect,[55] and physiological arousal[24,55]. During speech anticipation, degree of social anxiety is correlated with increased negative self-beliefs,[56] self-reported anticipatory anxiety,[56,57] and physiologic arousal[56,57]. During anticipation of giving a speech, activa-

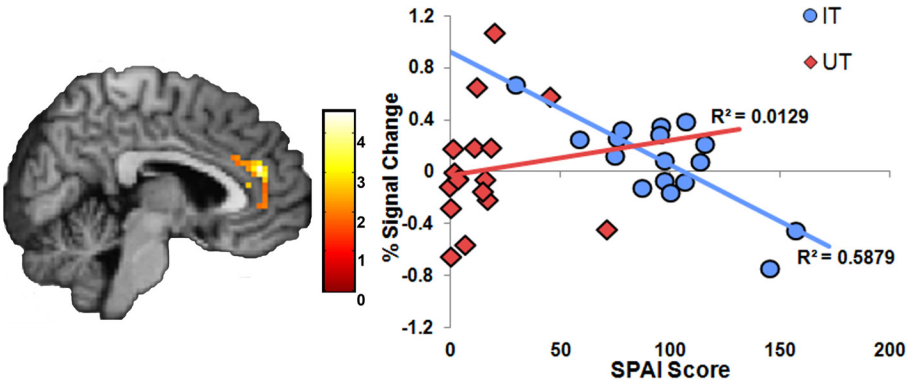

Figure 3: *Increased activation in the rostral anterior cingulate in subjects with fewer social anxiety symptoms.* During anticipation of fear faces, greater anterior cingulate activation in the inhibited temperament group (IT) was associated with fewer social anxiety symptoms (R2 = 0.59), but not in the uninhibited temperament group, (UT; R2 = 0.01).

tion is increased in regions associated with anticipation of aversive stimuli, including in the amygdala[24,43] and insula[43], and decreased in the dACC and dlPFC[43], in patients with social phobia, compared with controls. While studies of anticipation of "protect" against development of anxiety disorders; however, this has not been explicitly tested.

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Social phobia is defined as the fear or avoidance of one or more performance or social evaluative situations, especially those in which an individual is exposed to social evaluation or scrutiny by others. Symptoms of social phobia are often most prominent during the anticipation of social situations and is accompanied by heightened physiological arousal,[24,55] anticipatory anxiety,[24,55] and negative self-beliefs[56,57]. Understanding the neurobiology of anticipation of social situations has the potential to advance our understanding of the pathophysiology of social phobia.

Anticipation of aversive social stimuli in social phobia has typically been measured using a speech anticipation task, which reflects some of the common triggers of social phobia – fear of social evaluation. Subjects are given several minutes to prepare a speech on a given topic, and then they deliver the speech to a group of experimenters or in front of a video camera. Anticipation of public speaking increases state anxiety,[24,55] negative affect,[55] and physiological arousal[24,55]. During speech anticipation, degree of social anxiety is correlated with increased negative self-beliefs,[56] self-reported anticipatory anxiety,[56,57] and physiologic arousal[56,57]. During anticipation of giving a speech, activation is increased in regions associated with anticipation of aversive stimuli, including in the amygdala[24,43] and insula[43], and decreased in the dACC and dlPFC[43], in patients with social phobia, compared with controls. While studies of anticipation of public speaking show similar results to studies of aversive anticipation in other anxiety disorders, public speaking tasks do not target symptoms of the most disabling subtype of social phobia.

Social phobia has two subtypes: generalized and non-generalized. Generalized social phobia includes multiple social fears; non-generalized social anxiety disorder is confined to a single social fear (usually public speaking), is less disabling, and is rarely brought to medical attention[58]. Generalized social phobia is highly impairing and can result in fear of interacting with other people,[58] dropping out of school,[58] losing a job,[58] and psychiatric comorbidities, including other anxiety disorders and depression[59]. While studying the anticipation of public speaking is important in understanding non-generalized social anxiety disorder, giving talks in front of large groups of people can often be avoided. In generalized social anxiety disorder, encounters with unfamiliar individuals on a day-to-day basis cause impairment and anxiety, and cannot be avoided. Therefore, studying differences in brain activation to more common social stimuli, such as novel faces, is important in understanding the neural basis of generalized social phobia and to prevent and treat this disorder. One way to understand the pathophysiology of generalized social phobia might be to examine brain function during the anticipation of mildly aversive social stimuli.

One study by our lab (Clauss & Blackford, in preparation) has examined the neural correlates of anticipation of fear faces. To study social anxiety disorder, we study a high-risk group, individuals with an inhibited temperament. Inhibited temperament is associated with a 7-fold increased odds of developing social phobia.[60] In individuals with an inhibited temperament, anticipation of fear faces is associated with greater activation in the dlPFC and dACC, relative to those with an uninhibited temperament. In the inhibited

temperament group, dACC activation was negatively correlated with social phobia symptoms, as measured by the Social Phobia and Anxiety Inventory[61] (see **Figure 3**). The uninhibited group showed no significant change in activation from baseline during anticipation of faces, suggesting that an upcoming mild social stimulus evokes anticipatory activity in the inhibited temperament group only; additionally, there was no relationship between dACC activation and symptoms in the uninhibited temperament group (see **Figure 3**). These results suggest that anticipation of a mildly aversive social stimulus may be a unique probe for social anxiety symptoms.

Implications for Treatment

Anticipation of aversive stimuli is a key process in anxiety disorders and should be targeted in treatment of the disorders.[4] Neural responses during anticipation of aversive stimuli have been shown to be sensitive to treatment effects. Anxiolytic medications, including selective serotonin reuptake inhibitors and pregabalin, decrease insula and amygdala activity during anticipation.[62,63] Additionally, non-pharmacologic treatments are effective in modulating activity of emotion reactivity and regulation regions. In patients with social anxiety disorder[52], engaging in emotion regulation strategies during anticipation reduced insula and amygdala activity and increased ACC activity during anticipation and perception of aversive stimuli; in healthy controls, emotion regulation strategies also increased activity in the dlPFC and individual differences in emotion regulation were negatively correlated with amygdala activity during anticipation[25]. Greater pre-treatment anterior cingulate activation during anticipation in patients with generalized anxiety disorder was associated with better treatment response, suggesting that individuals who engage emotion regulation areas more at baseline may be more responsive to treatment.[11] Therapies that engage emotion regulation regions during aversive anticipation may be effective treatments for anxiety disorders.

Summary

Anticipation is a key psychological process and is highly adaptive by allowing individuals to avoid or modify upcoming aversive events. A network of brain regions is activated during anticipation of aversive stimuli, including the amygdala, insula, dorsal anterior cingulate cortex, and dorsolateral prefrontal cortex. In individuals with anxiety disorders, this network is disrupted; typically, activation of emotion reactivity regions is higher and activation of emotion regulation regions is lower. Anticipation of public speaking has been studied extensively in social phobia, but anticipation of more mild aversive social stimuli, such as single fear faces, has only been studied in a high-risk group. Considering the disability associated with generalized social phobia, which includes fear of daily social interactions, anticipation of more mild aversive social stimuli should be investigated and targeted for treatment. Enhanced activity of emotion regulation regions is associated with compensatory activity in high-risk, but high-functioning individuals, better outcomes, and better treatment response, suggesting that emotion regulation regions may be an important target for treatment of anxiety disorders.

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REVIEWS

The α1 Subunit in Sickness and in Health: Properties of the Most Predominant $GABA_A$ Receptor Subunit and Implications of Its Dysfunction in Human Epilepsy

Elizabeth Deel

The aim of this review is to highlight the role of the a1 subunit of the $GABA_A$ receptor under normal conditions and to examine the consequences of its dysfunction in the context of epilepsy. First, background information relevant to $GABA_A$ receptors is discussed, followed by a summary of the biophysical properties conferred by the a1 subunit and its developmental expression patterns. Next, key findings from a1 subunit knockout mice are reviewed. Lastly, the important role of the a1 subunit in regulating inhibitory tone in the CNS is highlighted by examining consequences of mutations in the a1 subunit implicated in generalized human epilepsy syndromes.

Keywords *GABA, $GABA_A$ receptor, heterogeneity, alpha-1 subunit, epilepsy*

Introduction

$GABA_A$ receptors ($GABA_A$Rs) are a family of chloride-selective, ligand-gated ion channels that mediate the majority of fast inhibition in the adult central nervous system[1]. $GABA_A$Rs belong to a larger superfamily of ligand-gated ion channels called Cys-loop receptors, which also includes nicotinic acetylcholine receptors, glycine receptors, and serotonin type III receptors[2,3]. The $GABA_A$R gene family is comprised of at least 19 different subunits which are classified by sequence homology into 8 subtypes. These subunits can assemble in various combinations to produce functional $GABA_A$Rs: α1-6, β1-3, γ1-3, δ, ε, π, θ, and ρ1-3[4]. Based on the large number of different $GABA_A$R subunits, there are seemingly myriad different subunit combinations possible, although only a subset of these theoretical combinations has been identified *in vivo*[5]. The cache of possible subunit combinations coupled with differing spatial and temporal expression patterns provides considerable structural and functional heterogeneity to $GABA_A$Rs. Distinct subunit combinations produce distinct receptor isoforms which display highly variable properties throughout development and in adulthood[6–9]. This topic will be discussed in further detail below.

The vast majority of $GABA_A$Rs exist as a combination of two α subunits, two β subunits, and either a γ or δ[5,10] subunit arranged as shown in **Figure 1A-B**. The ε, π, and ρ1-3 subunits are far less common and are generally positioned in place of the γ or δ subunit, while the θ subunit can assume the position of the β subunit. The morphology of each $GABA_A$R subunit includes several characteristic features. A large extracellular domain at the N-terminus contains the characteristic disulfide bridge between two cysteine residues that creates the "Cys-loop" for which the receptor family is named. There are four helical transmembrane domains termed M1-M4, with the M2 of each subunit lining the ion pore. conntecting M3 and M4, there is a large intracellular domain, and beyond M4 is a very small extracellular C-terminal domain (**Figure 1C**)[1,2,11].

Full activation of $GABA_A$Rs requires the binding of two molecules of the neurotransmitter γ-amino butyric acid (GABA)—one at each α/β subunit interface[12,13]. Upon activation of the receptor, the channel opens and chloride (Cl^-) flows down its electrochemical gradient through the pore. In the mature brain, this leads to an influx of Cl^-, which causes the membrane potential of the cell to hyperpolarize and thus inhibits the generation of action potentials.

As previously mentioned, the wide variety of $GABA_A$R isoforms supports extensive functional heterogeneity, perhaps best demonstrated by the existence of two distinct forms of $GABA_A$R-mediated inhibition: tonic and phasic[14]. Tonic inhibition is mediated by extrasynaptically localized $GABA_A$Rs largely comprised of a δ subunit with an α4 and/or an α6 subunit[15], though some extrasynaptic $GABA_A$Rs containing α5 and lacking a δ subunit are known to func-

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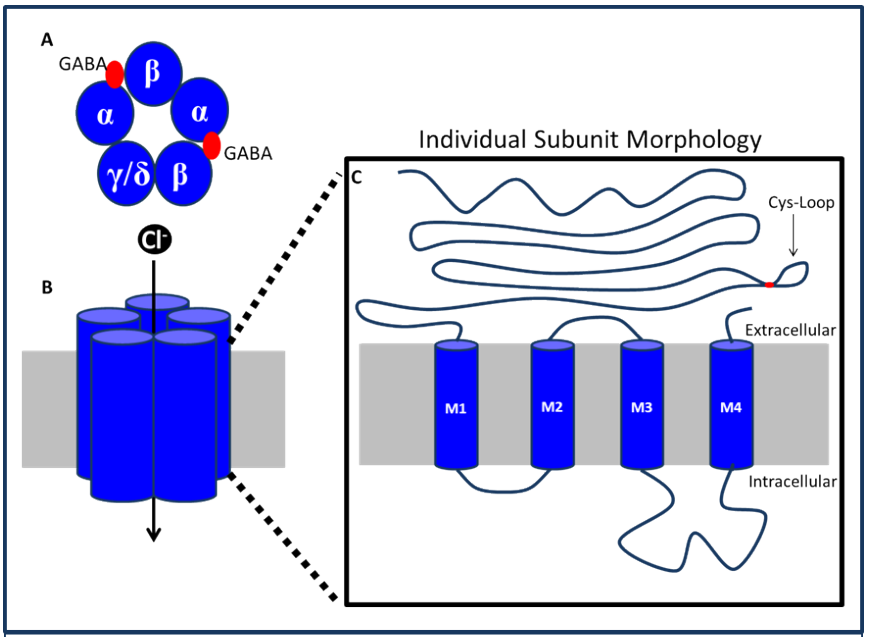

Figure 1: *Structure of $GABA_A$ receptors.* A: Top down view of typical subunit arrangement with GABA binding site indicated by red ovals. B: Side view of receptor depicting chloride flowing through the pore. C: Morphology of an individual subunit.

tion extrasynaptically as well[16]. Extrasynaptic $GABA_A$Rs are continuously activated by low levels (1M) of ambient GABA typically overflowing from the synaptic cleft. The properties of these receptors are conducive to persistent extrasynaptic activation, as they are highly sensitive to GABA, activate relatively slowly, and desensitize minimally[17]. The functional role of tonic inhibition seems to be the regulation of neuronal excitability. Conversely, phasic inhibition is mediated by synaptic $GABA_A$Rs, which most often contain a γ subunit with α1, α2, and/or α3 subunits. Unlike their extrasynaptic counterparts, synaptic $GABA_A$Rs are transiently activated by much higher (1mM) concentrations of GABA released from the presynaptic neuron into the synaptic cleft. In contrast to extrasynaptic $GABA_A$Rs, synaptic $GABA_A$Rs mediate tonic inhibition to allow for fast transmission of a fleeting signal and are thus less sensitive to GABA, activate rapidly, and desensitize extensively[18]. The short-lived activation of these receptors produces a transient inhibitory post-synaptic current (IPSC) which-translates presynaptic GABA release into a post-synaptic signal[19,20]. The remainder of this review will focus on the most predominant subtype of synaptic receptors: those containing the α1 subunit. The distinct receptor properties and known roles of the α1 subunit, as well as important findings from α1 subunit knockout mice and recently discovered epilepsy-associated mutations in the α1 subunit are discussed below.

Biophysical Properties of the α1 Subunit Relative to Other Synaptic α Subunits

The α1 subunit is the most predominant α subunit in the adult brain and is most often assembled into the α1β2γ2 $GABA_A$R isoform comprising 60% of all $GABA_A$Rs[8]. Further heterogeneity exists among synaptic receptors. The specific α subtype influences biophysical properties of $GABA_A$Rs including GABA sensitivity and the rates of activation, deactivation, and desensitization[21–25]. The primary strategy employed to determine the contribution of each α subunit to a particular property is to express different α subunits with the same β and γ subunit partners in heterologous cells and explore the properties of interest. One such property, GABA sensitivity, is defined by the concentration of GABA that is required to produce a given response. A common measure of GABA sensitivity is the concentration of GABA required to elicit a half-maximal response in a given receptor subtype, known as the EC50. A low EC50 indicates higher sensitivity and vice versa. The activation rate of a particular $GABA_A$R is the rate at which receptor current increases from 10% to 90% of the maximal or peak current, and the deactivation rate is the rate at which current amplitude decreases after GABA is removed.

The desensitized state of the receptor is a high-affinity state in which GABA is bound but the ion channel is closed. The desensitization rate is the rate at which the response diminishes in continued presence of GABA. Each of the biophysical properties defined above influence the shape and time course of $GABA_A$R-mediated IPSCs. Thus, the identity of the α subunit within the receptor in large part

Table 1: Relative Biophysical Properties Conferred by Synaptic α Subunits	
GABA Sensitivity	α1 > α2 > α3
Activation Rate	α2 > α1 > α3
Deactivation Rate	α1 > α2 > α3
Desensitization Rate	α1 = α2 > α3

Table 1: *Biophysical properties by synaptic α subunits.*

dictates the properties of inhibitory currents. The biophysical properties of α1 containing $GABA_A$Rs relative to other synaptic α subunits are summarized in **Table 1**[21–24].

Interestingly, the properties of $GABA_A$R mediated IPSCs are known to change throughout development[26,27]—namely the decay kinetics which are heavily influenced by the deactivation and desensitization rates of the $GABA_A$Rs[28,29]. In fetal and early postnatal development, $GABA_A$R-mediated IPSCs decay relatively slowly; later in development, the IPSCs decay much more rapidly. The timing of this change coincides with the timing of a well-established developmental change in $GABA_A$R α subunit expression[6,30,31]. Early in development, the α2 and α3 subunits predominate, but soon after birth their expression begins to wane while the expression of the α1 subunit steadily increases to become the most abundant α subunit by postnatal day 12 in mice[6]. A comparison of juvenile and mature $GABA_A$R-mediated IPSCs is shown in **Figure 2**. Given that the identity of the α subunit impacts IPSC properties, it is feasible that the developmental changes in α subunit expression and IPSC decay kinetics are causally linked. Indeed, it has been shown that in mice lacking the α1 subunit juvenile IPSC kinetics persist into adulthood[32–34]. The functional role of this developmental switch in α subunit expression and the concomitant change in IPSC kinetics is currently not well understood.

Findings From α1 Subunit Knockout Mice

In 2001, transgenic mice lacking the α1 subunit of the $GABA_A$R exhibited a 50-60% decrease in the total number of $GABA_A$Rs in the brain[33,35]. Consistent with this finding, the expression of the β2/3 and γ2 subunits—the most common binding partners of the α1 subunit—is also decreased in α1 knockout mice[36,37]. Given that the α1 subunit is the most abundant subunit and its loss results in a loss of the majority of $GABA_A$Rs in the brain, it is very surprising that these animals are viable and lack any obvious phenotypic abnormalities aside from a slight handling-induced tremor. The fact that the mice are overtly normal could suggest that changes occur within the $GABA_A$R system to compensate for the loss of α1. Indeed, post-transcriptional increases in the expression of the other α subunits have been observed[36,37], but the nature and extent of the compensation seems to vary among brain regions and has not been systematically quantified in the entire brain.

One study suggests that neurons upregulate the subunits they normally express rather than expanding their subunit repertoire[36], which is consistent with a post-transcriptional mode of upregulation. The consequences of these compensatory changes are not completely understood, but α1 knockout mice fail to develop mature IPSC kinetics[32–34] and exhibit a lower threshold for pharmacologically induced seizures[38]. This phenotype could indicate a decrease in inhibitory tone, although it was reported that α1 knockout mice did not experience spontaneous seizures[35]. However, it is important to note that these conclusions were drawn by visual inspection only rather than EEG analysis. Certain types of seizures, such as absence seizures, result in very subtle alterations in behavior that are difficult to detect even in a human, much less in a mouse. Thus it is possible that these animals did have seizures but did not display any easily detectable seizure behavior.

The α1 Subunit and Epilepsy

Because $GABA_A$Rs are the primary source

Electroencephalogram (EEG):
Method to measure electrical activity of the brain using electrodes on the surface of the scalp.

Missense Mutation:
Non-synonymous point mutation in which the identity of a single nucleotide is changed resulting in a codon that codes for an amino acid that differes from that of the WT protein at that particular location.

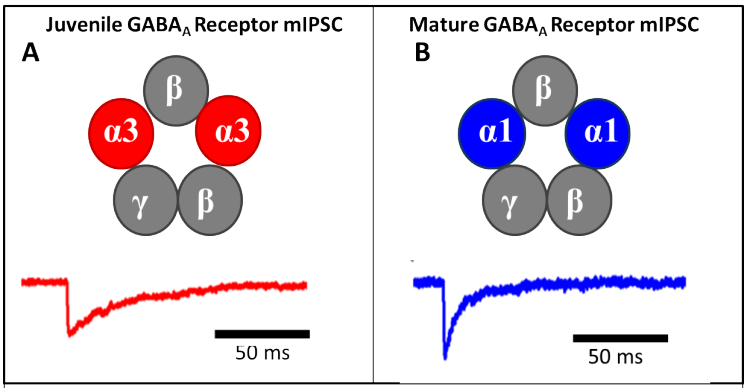

Figure 2: *Developmental change in α subunit expression and IPSC kinetics.* A: Juvenile α3 containing receptor with much slower IPSC delay kinetics. B: Mature α1 containing receptor with fast IPSC decay kinetics.

Endoplasmic Reticulum Associated Degradation:
A cellular process that marks misfolded proteins within the ER for ubiquitination followed by degradation via the proteasome.

Nonsense Mediated Decay:
Cellular mechanism to detect nonsense mutations (premature stop codons) and prevent translation of truncated proteins by degrading the mutant mRNA.

of inhibition in the central nervous system, it is not surprising that several mutations in various GABA$_A$R subunits have been identified in patients with idiopathic generalized epilepsy (IGE) syndromes such as childhood absence epilepsy and juvenile myoclonic epilepsy[39–41]. Epilepsy is a large collection of syndromes diagnosed upon the occurrence of two or more unprovoked seizures. Epilepsy may be classified as IGE if the cause is thought to be genetic and the seizures appear to involve the entire brain simultaneously with no obvious focal origin. Most IGEs are thought to be multigenic, which renders developing animal models fairly difficult. However, the identification of monogenic forms of IGE has permitted the generation of animal models based on human disease-associated mutations. To date, there have been four mutations identified specifically in the α1 subunit in human patients suffering from idiopathic generalized epilepsy (IGE.) Two of these mutations, K353delins18X and D219N[42], were only recently reported and have not been fully characterized. The other two however, A322D[43] and S326fs328X[44], have been extensively studied.

α1(K353delins18X) Mutation

The α1(K353delins18X) mutation was identified in 1 unaffected and 3 affected individuals with IGE exhibiting generalized tonic-clonic seizures. These seizures manifest as a sudden tensing of skeletal muscles followed by rapid contractions and relaxations resulting in characteristic convulsions[45]. This mutation involves the insertion of 25 base pairs into intron 10 which interrupts splicing and causes the retention of intron 10 in the transcript. The inclusion of intron 10 leads to an 18-amino acid insertion into the protein as well as the truncation of the fourth transmembrane domain due to a premature stop codon. Work in heterologous expression systems revealed that the protein is localized to the ER with complete loss of cell surface expression. In agreement with these findings, GABA-mediated currents were absent in these cells[42]. The fate of the mutant protein and the mechanism by which the α1(K353delins18X) mutation leads to epilepsy is yet to be determined.

α1(D219N) Missense Mutation

The D219N missense mutation was identified in 4 of 5 affected individuals with IGE in a French-Canadian family exhibiting IGE or febrile seizures(FS)—so aptly named due to their coincidence with fever[45]. Two of the four individuals with FS also reported a single generalized tonic clonic seizure. Studies conducted in heterologous expression systems indicate that surface expression of the mutant subunit is reduced as compared to WT α1 subunit, consistent with observations of decreased GABA-evoked peak current amplitudes. Additionally, α1(D219N) subunit-containing receptors were shown to desensitize more rapidly than WT α1 subunit-containing receptors[42]. Further studies characterizing the effects of this mutation both *in vitro* and *in vivo* will be required to elucidate the mechanism by which it promotes the development of epilepsy.

α1(A322D) Missense Mutation

The A322D missense mutation was identified in 8 affected individuals within a large French-Canadian family suffering from a type of IGE called juvenile myoclonic epilepsy (JME). Myoclonic seizures are characterized by sudden, brief, involuntary jerks of the arms or legs[45]. This mutation is autosomal dominant and results in the insertion of a charged aspartate residue in place of a highly conserved alanine within the M3 domain[39–41,43]. Experiments in heterologous cells indicate that this mutation disrupts the insertion of M3 into the lipid bilayer which results in its retention in the endoplasmic reticulum (ER) and subsequent partial degradation through ER-associated degradation[39–41,46] (ERAD). However, the mutant subunit is not completely degraded, as total and surface mutant protein is detectable, albeit at significantly lower levels than the WT α1 subunit[39–41,46]. It has also been postulated that the α1(A322D) mutation exerts a dominant negative effect by oligomerizing with and trapping WT subunits in the ER which are then subject to ERAD[39,47]. Consistent with reduced surface expression, the peak GABA-evoked current through receptors containing the α1(A322D) subunit was reduced 88%[47]. In the mutant receptors, open time of the channel was considerably reduced. Additionally, α1(A322D) subunit-containing receptors exhibited substantially reduced sensitivity to GABA with a nearly 100-fold increase in the GABA EC50[39,40]. It would be particularly enlightening to study the effects of this mutation *in vivo* and to that end, our group is in the process of generating an α1(A322D) knock-in mouse line.

α1(S326fs328X) Frameshift Mutation

The α1(S326fs328X) mutation is an autosomal dominant *de novo* mutation identified in a patient with childhood absence epilepsy. In contrast to the previously mentioned seizure types, absence seizures are not associated with any sort of convulsions or motor movements. Rather, they are characterized by sudden brief lapses in consciousness often accompanied by a blank stare[45]. A single base pair deletion leads to a frameshift and premature termination codon (PTC) in the eighth exon which corresponds to the third transmembrane domain of the protein[44]. The PTC has been shown to induce nonsense mediated decay (NMD) of the mutant mRNA, albeit incomplete. Mutant mRNA that escapes NMD is subsequently translated into truncated protein which is retained in the ER and subjected to ERAD[48,49]. Thus, the α1(S326fs328X) mutant subunit is not incorporated into the cell membrane and GABA-evoked currents are absent[44,48].

Based on these findings, it is thought that the epilepsy phenotype is a result of haploinsufficiency in the WT α1 gene. This raises the intriguing possibility that heterozygous α1 knockout animals could serve as a model for this disease. Indeed, our group recently revealed through EEG analysis that heterozygous α1 knockout mice do in fact experience seizures, though they are absence seizures rather than convulsive seizures[50]. This may explain why it was previously reported that α1 knockout mice did not exhibit an epileptic phenotype as mentioned above. The seizures were greatly attenuated by treatment with ethosuximide (ETX), the most commonly prescribed drug for absence seizures in human patients. These findings represent a novel model of absence epilepsy based on a mutation identified in a human epilepsy patient.

Conclusions

$GABA_A$Rs are a heterogeneous population of receptors and their properties are heavily influenced by their α subunit composition. The α1 subunit is the most predominant subunit in the adult brain and is largely responsible for the maintenance of inhibitory tone in the CNS. As evidenced by the consequences of the dysfunction or loss of the α1 subunit, it also seems to be involved in epilepsy susceptibility. The transgenic α1(A322D) knock-in and α1 knockout mouse lines that our group focuses on represent the first mouse models of genetic epilepsy based on mutations in the α1 subunit identified in human epilepsy patients. These two mutations were both identified in patients suffering from generalized epilepsy, but their seizure phenotypes were distinct. Based on our preliminary analyses, the epileptic phenotypes of the two mouse lines also differ. By dissecting the similarities and differences in pathogenesis behind these two models, we aim to identify common themes among all generalized epilepsies and also delineate important differences that contribute to distinct disease manifestations. While epilepsy is an exceptionally complicated and heterogeneous condition, the advent of animal models reflecting specific disease-associated mutations in $GABA_A$Rs represents a promising and riveting new avenue for advancing our understanding of the pathogenesis of generalized epilepsy.

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Further information: http://www.mc.vanderbilt.edu/neurology/faculty/gallagher.htm

CANDIDATE
REVIEWS

Amygdala Developmental Consequences of Childhood Maltreatment

E. Kale Edmiston

Childhood maltreatment is a significant social problem associated with increased risk for depression and anxiety disorders. Despite this, many adults who experience childhood maltreatment do not develop psychiatric disorders and the neurobiological correlates for risk and resilience following childhood maltreatment are not well understood. Even in the absence of psychiatric diagnosis, childhood maltreatment can be associated with sub-clinical alterations in threat detection, a likely adaptive response to the early environment. Enhanced threat sensitivity following childhood maltreatment may be mediated by alterations in the developmental trajectory of the amygdala, a region often associated with threat detection. Functional MRI studies of populations with childhood maltreatment histories suggest heightened amygdala activation to aversive stimuli. Likewise, rodent models of chronic early stress exposure suggest amygdala effects, including increased dendritic arborization and decreased expression of the serotonin transporter (5-HTT). Genetic studies have also implicated 5-HTT, reporting an increased risk for depression following childhood maltreatment in carriers of the low-expressing of the 5-HTTLPR. Neuroimaging genetic studies have found increased amygdala BOLD signal in response to emotional or threatening stimuli in the low-expressing allele carriers exposed to maltreatment. Taken together, this literature suggests that the amygdala has heightened reactivity to threatening stimuli in people exposed to childhood maltreatment, and that these effects may be mediated by genetic variations in the serotonin system. Importantly, enhanced risk for depression is an end-point outcome well downstream of a host of genetic and environmental factors that interact dynamically throughout development. Future studies should address possible mediating factors by assessing for onset and duration of maltreatment, symptom severity, as well as gene x environment effects.

Keywords: *fMRI, serotonin transporter, childhood maltreatment, amygdala, anxiety, depression*

Introduction

Childhood maltreatment is a significant social problem affecting 3.7 million children annually in the United States alone[1]. Childhood maltreatment, which can include physical, emotional, and sexual abuse, as well as physical and emotional neglect, is associated with a host of negative outcomes, including increased risk for psychiatric disorders such as major depressive disorder and anxiety disorders[2]. Even in the absence of psychiatric symptoms that meet criteria for diagnosis, populations exposed to maltreatment can experience sub-clinical alterations in emotional processing, such as increased threat sensitivity and decreased emotional regulation[3-5]. Emotional processing changes are likely adaptive responses to early environmental exposure to threat, whereby threatening experiences have become generalized to the broader social environment as a way to avoid additional harm[6]. Observed changes in threat sensitivity and emotional regulation are likely mediated by the effects of early life stress on the developmental trajectory of the amygdala, a region implicated in threat detection and fear conditioning[7], as well as anxiety and mood disorders[8]. Despite increased risk for psychiatric disorders and behavioral dysfunction in populations exposed to childhood maltreatment, a significant proportion do not develop negative outcomes later in life. What, then, are the neurobiological correlates of enhanced risk for poor outcome following childhood maltreatment? This review will examine potential gene x environment developmental effects of childhood maltreatment on the structure and function of the amygdala, as well as how altered amygdala development may mediate increased risk for psychiatric disorder.

Amygdala Alterations in Magnetic Resonance Imaging Findings of Childhood Maltreatment

Enhanced sensitivity to potentially threatening emotional

Reference	Imaging Paradigm	Maltreated/ Total	Age Range	Psychiatric Diagnoses	Maltreatment Type	Maltreatment Assessment	Amygdala Findings
Garret et al., 2012	emotional face passive viewing	30/56	10-16	None	PN, EN, PA, EA, SA	Self report	BOLD increase, neutral and angry faces
Grant et al., 2010	emotional face flanker task	10/36	18-55	MDD	PN, EN, PA, EA, SA	Self report	BOLD increase, negative affect faces
Maheu et al., 2010	emotional face directed viewing	11/30	9-18	Anxiety d/o, n=2	PN, EN	Social services documentation	BOLD increase, angry and fear faces
McCrory et al., 2011	emotional face passive viewing	20/43	10-13	None	PN, PA, EA, SA	Social services documentation	BOLD increase, angry faces
Protopopescu et al., 2005	traumatic word passive viewing	9/14	20-55	PTSD	PA, SA	Self report	BOLD increase, traumatic words
Tottenham et al., 2011	emotional face go/no-go task	22/44	7-13	Anxiety d/o, n=2; ADHD, n=5; ODD, n=1	Previous institutionalization	Social services documentation	BOLD increase, fear and distractor faces
Van Harmelen et al., 2012	emotional face passive viewing	60/135	33-41	MDD or Anxiety d/o	EN, EA	Self report	BOLD increase for all faces
Bremner et al., 1997	sMRI, hand tracing	17/34	25-52	PTSD	PA, SA	Clinician interview	No volumetric differences
De Bellis et al., 2001	sMRI, hand tracing	9/18	8-12	PTSD	SA	Social services documentation	No volumetric differences
Driessen et al., 2000	sMRI, hand tracing	21/42	22-35	BPD	PN, EN, PA, EA, SA	Self report	Volumetric decrease
Edmiston et al., 2011	sMRI, VBM	42*	12-17	None	PN, EN, PA, EA, SA	Self report	Volumetric decrease correlated with frequency
Thomaes et al., 2010	sMRI, VBM	31/59	22-47	PTSD	PA, SA	Clinician interview	No volumetric differences
Tottenham et al., 2010	sMRI, automated segmentation	34/62	5-15	Anxiety d/o, ADHD, ODD,	Previous institutionalization	Social services documentation	Volumetric increase for late adopted relative to early adopted and control
Van Harmelen et al., 2010	sMRI, VBM	84/145	35-39	MDD or Anxiety d/o	EN, PA, EA, SA	Clinician interview	No volumetric differences

Table 1: *Summary of MRI literature in populations with childhood maltreatment exposure.* fMRI, functional magnetic resonance imaging; sMRI, structural MRI; VBM, voxel-based morphometry; PTSD, post-traumatic stress disorder; MDD, major depressive disorder; ADHD, attention deficit hyperactivity disorder; ODD, oppositional defiant disorder; PN, physical neglect; EN, emotional neglect; EA, emotional abuse; SA, sexual abuse.

*individual differences regression analysis

faces is a consistent finding in adults[9], adolescents[10], and children[11-13] exposed to childhood maltreatment. In one study, participants with maltreatment demonstrated increased sensitivity to angry faces during an emotional face-morphing paradigm. Participants viewed a set of faces that morphed along a continuum from happy to fear, happy to sad, angry to fear, or from angry to sad and were asked to identify the emotion when presented with a pair of images from each continuum. Participants with a maltreatment history overrated facial expressions as angry and identified angry facial expressions at a lower intensity than participants that did not experience maltreatment, suggesting enhanced sensitivity to potential threat, with discrimination of potential threat at a reduced sensory threshold and with decreased reaction time[14]. Furthermore, a study of adolescents exposed to childhood physical abuse suggests increased attentional allocation to threatening or aversive imagery compared to neutral or positive imagery during an emotional dot-probe task[15]. Taken together, the behavioral literature suggests that emotional stimuli are particularly salient to maltreated populations, and that social information, such as faces may be of particular importance given the social nature of early maltreatment. Although adaptive in the context of a threatening upbringing, this enhanced attention to threatening stimuli may mediate increased risk for mood and anxiety disorders later in life[15-16].

Changes in amygdala structure and function are candidate mediators of the enhanced salience of emotional stimuli secondary to maltreatment. Previous functional magnetic resonance imaging (fMRI) studies of PTSD populations exposed to trauma, although not necessarily childhood maltreatment, have implicated the amygdala in emotional processing, and have also shown correlations between increased amygdala activation and PTSD symptom severity (for review, see 17-18). Likewise, fMRI studies of adults with childhood maltreatment exposure have found heightened amygdala activation to threatening or emotional stimuli, including negatively valenced emotional faces[19-20] and traumatic reminder words[21] (**Table 1**). FMRI studies in children and adolescents report similar findings, with increased amygdala BOLD signal for negatively valenced emotional faces the most often-reported finding[22-25]. Interestingly, although all of these studies report increased BOLD signal, several have reported heightened amygdala reactivity to both neutral and emotional facial expressions[20,25]. This discrepancy could be due to a generalized enhancement in threat detection, similar to what has been reported in the behavioral literature, such that neutral faces are perceived as potentially threatening. However, there is also evidence that in typically developing children there is no heightened amygdala activation to fear faces relative to neutral faces, as there is in adults[26], suggesting that height-

ened amygdala reactivity to fear faces in maltreated child and adolescents samples may reflect an altered developmental trajectory. In addition to alterations in amygdala BOLD signal magnitude, there is also evidence for changes in amygdala BOLD signal time course. Using an emotional face block design, Garret et al. report that increases in amygdala BOLD are greatest relative to control subjects in the early rather than the late phase for angry and fearful faces, suggesting that maltreated subjects may be primed for potential threat[25]. Taken together, the fMRI literature suggests that heightened amygdala activation to emotional stimuli may underlie the increased threat sensitivity observed in behavioral studies of maltreated populations, as well as their increased risk for mood and anxiety disorders.

Volumetric MRI studies of the amygdala in childhood maltreatment have been less consistent than the fMRI literature (**Table 1**). Voxel-based morphometry studies of the amygdala in adults have found decreases[27] or no differences[28-30] in amygdala volume, but are generally confounded by the study of participants with psychiatric diagnoses, such as PTSD and Borderline Personality Disorder, that are also associated with amygdala changes. Studies of children and adolescents have also been mixed, with reports of increases[31], decreases[32], and no differences[33] in amygdala volume. Mixed findings may in part be due to the still-developing nature of limbic-prefrontal circuits in adolescence, such that early stress exposure may trigger precocious amygdala development and sensitization, followed by volumetric decreases[34]. Furthermore, the issue of risk vs. resilience confounds studies of adolescents or children with maltreatment exposure and no current psychiatric diagnosis. Some adolescents may still develop a mood or anxiety disorder in adulthood, but it is also possible that a study of young adults or late adolescents without psychiatric disorders has sampled the most resilient members of the maltreated population; the reported amygdala volume reductions could be a marker of resistance to later mood or anxiety symptoms, thereby making conclusions about the structure of the amygdala in at-risk populations difficult. However, a recent study using an individual differences approach found a negative correlation between reported maltreatment severity and amygdala volume in adolescents, particularly in emotional maltreatment, suggesting that part of the heterogeneity of previous findings may be due to maltreatment type and the limited sensitivity of group comparison studies to detect potential biologically significant thresholds in maltreatment severity[32]. An important paper by Tottenham et al. assessed a group of orphans who experienced early childhood institutionalization in Romania, followed by adoption in the United States. In this study, there were no overall differences in amygdala volume between previously institutionalized children and controls. However, further comparison of early versus late adopted subjects revealed a significant correlation between amygdala volume and age of adoption, such that later adopted children had larger amygdala volumes than early adopted children[31]. Thus, age of maltreatment onset, duration of maltreatment, and age of assessment are all potential mediators of amygdala volumetric findings; future studies with an individual difference-based approach may prove more helpful than group comparison analyses in teasing apart the relative importance of the timing, severity and duration of maltreatment on amygdala development.

Amygdala Alterations in Rodent Models of Early Life Stress

Rodent models of early life stress effects on the amygdala have been more consistent than the human subjects literature. Both juvenile and adults rodents exposed to chronic immobilization stress have increased dendritic arborization of pyramidal neurons in the basolateral amygdala, in contrast to cells in the CA3 region of the hippocampus, which typically show dendritic atrophy. These chronically stressed rodents also show increased anxiety and depressive -like phenotypes as measured in behavioral tasks such as the elevated plus maze[35] and forced swim task[36]. Other rat studies have employed maternal separation paradigms, a model of early life stress in rodents, where pups are separated from their mothers for long or short periods of time. One maternal separation study has shown that the long-separated rats exhibited down-regulation of the serotonin transporter (5-HTT) as well as of inhibitory 5-HT_{1A} receptors in the amygdala. Long-separated rats also demonstrated an anxiety phenotype compared to short-separated rats, as assessed by the open field test[37]. Other rodent studies of chronic glucocorticoid administration have suggested down-regulation of the 5-HT_{1A} receptor subtype and up-regulation of 5-HT_{2A} receptor subtype[38]. It is thought that in limbic regions, activation of 5-HT_{1A} receptors may be anxiolytic, while serotonergic innervation of 5-HT_{2A} receptors may be anxiogenic (for review, see 39). These region and receptor subtype specific alterations may explain the heightened amygdala reactivity common in populations exposed to early life stress.

Rodent models are better able to assess differential effects of chronic versus acute stress than human studies. In a rodent study employing both chronic and acute immobili-

zation stress, chronic stress was associated with increased dendritic arborization in the basolateral amygdala the day after stress termination. In contrast, after a single acute stress exposure, increased dendritic spine density was observed in the basolateral amygdala, but only after a delay period of ten days[40]. Given the role of the amygdala in fear conditioning, this is likely an adaptive mechanism, allowing for priming for future potential stressors after early environmental uncertainty. Taken together, the rodent literature suggests increases in dendritic arborization that are specific to the amygdala after early stress; these changes may be secondary to alterations in serotonin receptor subtype density caused by excessive glucocorticoid exposure. Although caution is important when making inferences about human MRI literature on the basis of rodent models, it is possible that the heterogeneity in findings in the human literature is due to varying durations between maltreatment and assessment, or differences between chronic and acute stress effects.

5HTTLPR x Stress Effects on Depression Risk

Both the serotonin-mediated anxiety phenotypes in rodent models of early stress exposure, as well as the use of selective serotonin reuptake inhibitors (SSRIs) in depression treatment, suggest a 5-HTT mediated mechanism for observed functional and structural changes in the amygdala secondary to childhood maltreatment. The serotonin transporter is involved in the regulation of synaptic serotonin via reuptake of serotonin from the synapse into the presynaptic cell. The 5-HTT gene-linked polymorphic region (5-HTTLPR) is located on the 5' regulatory area of the serotonin transporter gene and has been of particular interest in the study of increased risk for depression in the presence of early life stress[41]. The 5-HTTLPR, originally thought to have a biallelic expression, has recently been shown to have a triallellic expression, with a short and long form allele. The long form allele has an L_G and L_A form, such that the L_G form is functionally similar to the low-expressing short or "S" allele. The S or L_G alleles are dominantly expressed and result in reduced transcription of SLC6A4A and decreased presence of 5-HTT at the synaptic membrane[42].

A landmark paper by Caspi et al. employed a population-based methodology to assess for a gene x environment relationship on depression rates. Their findings suggest an increased rate of depression for adult carriers of the S allele of 5-HTTLPR who also reported exposure to stress[43]. This study is one of the first studies to demonstrate a gene x environment effect of enhanced risk for a psychiatric disorder. However, there has been much controversy surrounding the reported observation, as some studies have been unable to replicate the 5-HTTLPR x stress finding[44-47]. This may be due to differences in stress assessment between studies. For example, types of stress exposure assessments vary wildly across studies; some have employed participant interviews both with and without medical record confirmation of maltreatment, which may be subject to retrospective bias. Others have used questionnaires to asses for stress exposure, ranging from brief, four question assessments for the presence or absence of physical or sexual abuse history[45,47], to detailed multi-item questionnaires that assess for the severity, timing, and duration of a host of different early life stressors[48-49]. Importantly, many of these studies have assessed for only physical or sexual abuse, which, although associated with an increased risk for later psychopathology, may be less salient than emotional maltreatment for both increased risk of later psychopathology[3,50] and amygdala volume alterations[32]. Furthermore, later studies of the 5-HTTLPR polymorphism have employed a triallelic analysis of the polymorphism, whereas other, earlier studies employed a biallelic (short or long) analysis[48,51-53]. Although the original Caspi et al. paper employed a biallelic analysis, differences in the underlying distribution of the L_g vs. L_a allele that were not assessed may account for heterogeneity of findings in the biallelic literature. Finally, recent studies have reported a sex x genotype x environment effect on 5-HTTLPR polymorphism and increased risk for depression, with only women with the low-expressing allelic variants showing enhanced risk for depression in the presence of childhood maltreatment[54]. This may explain the failure to replicate in studies that did not consider sex difference effects. Despite the heterogeneity of findings and methods in the literature, recent meta-analysis has suggested a positive finding, although with a smaller effect size than the original Caspi report[55].

5HTTLPR x Stress Effects on Amygdala Activation

Given the controversy regarding the enhanced risk for depression in short allele carriers with early life stress exposure, more recent literature has examined the effect of gene x environment on amygdala activation using fMRI. This literature has consistently found increased BOLD signal to emotional or threatening stimuli in the low-expressing allele carriers exposed to maltreatment[55]. Other studies have found decreased functional coupling of the amygdala with prefrontal regions, such as the anterior cingulate, which are thought to down regulate amygdala activity during emotional processing[56]. These findings suggest that the amyg-

dala has a heightened reactivity to emotional or potential threatening stimuli in low expressing carriers exposed to childhood maltreatment, and/or that there is decreased functional coupling between the amygdala and prefrontal cortex in this population. Combined genetic neuroimaging studies may be more fruitful in the study of risk for psychiatric disorder. Enhanced risk for depression is an end-point outcome well downstream of a host of genetic and environmental factors that interact dynamically throughout development; diagnostic categories are heterogeneous and depressive symptoms are likely the result of multiple potential biological causes and diagnostic categories. Therefore, by examining the association between genotype and intermediate phenotypes, such as BOLD signal or well-defined clinical subgroups, studies are better able to explore possible mechanisms for enhanced risk for depression by focusing on a single (or cluster of related) biological factors.

Concluding Remarks and Future Directions

MRI findings in amygdala volume and activation following childhood maltreatment suggest that this brain region is a significant contributor to emotional and behavioral alterations observed in maltreatment-exposed adults and children, including increased risk for mood and anxiety disorders. However, this literature is varied and complex due to a variety of factors that may moderate amygdala development, including 5-HTTLPR genetic polymorphisms and sex, as well as timing, duration, and type of maltreatment exposure. Future studies should address these possible interactive effects by assessing for severity and type of maltreatment, as well as for gene x environment interactive effects. Understanding the mechanism behind observed gene x maltreatment effects for depression risk will likely involve a close examination of 5-HT system alterations across development. Importantly, a better understanding of 5-HT system changes has the chance to improve clinical treatment of adult patients with maltreatment history and depression, as some studies have suggested that these patients, as well as patients with the low-expressing 5-HTTLPR allele, may be poor-responders to SSRI treatment[57-58]. Variations in treatment response may be due to underlying differences in serotonergic system function in patient subpopulations with the 5-HTTLPR risk allele and/or with childhood maltreatment. As study of brain alterations following childhood maltreatment is necessarily difficult for ethical reasons in humans, rodent models of early life stress are particularly important in dissociating the varying developmental effects of different types of maltreatment and maltreatment duration on amygdala structure and function. Translational research to assess potential alterations in 5-HT receptor density and mechanisms underlying resultant structural and functional remodeling of limbic circuits is necessary to inform observed changes in the human behavioral, genetic, and neuroimaging maltreatment literature.

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G Protein-Coupled Receptor Kinases (GRKs) and G Protein-Coupled Receptors (GPCRs): GRK6 as a Potential Drug Target for CNS Disorders

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G protein-coupled receptor kinases (GRKs) specifically interact with G protein-coupled receptors (GPCRs) and play an important role in terminating agonist-induced GPCR signaling. Activated by an agonist-stimulated GPCR, GRKs in turn phosphorylate the GPCR, which leads to recruitment and activation of β-arrestin and subsequent receptor desensitization. Through activation of β-arrestins, GRK can also participate in downstream signaling events. GRK6, one of the GRK isoforms, is expressed abundantly in the brain. In recent years, potential links between GRK6 and central nervous system (CNS) disorders, in particular Parkinson's disease (PD), have been suggested. Specifically, GRK6 appears to play a significant role in dopamine-mediated locomotor activity and dopamine agonist-induced dyskinesia. Therefore, GRK6 should be considered as a potential drug target for CNS disorders.

Keywords: *GPCR, GRK, GRK6, Parkinson's disease, dopamine, L-DOPA, dyskinesia*

Mechanism and regulation of GPCR signaling

GPCRs, such as rhodopsin, prostaglandin E_2 receptors and β adrenergic receptors, are members of the seven transmembrane receptor family. G proteins are heterotrimeric complexes comprised of G_α and $G_{\beta\gamma}$ subunits. Upon agonist binding to a GPCR, the G_α subunit exchanges its bound GDP with GTP, which triggers release of G_α and its subsequent association with effectors. The released $G_{\beta\gamma}$ subunit can also stimulate downstream signaling[1]. GPCR signaling is controlled by receptor desensitization, a process that involves receptor phosphorylation by G protein-could receptor kinases (GRKs) and subsequent β-arrestin binding[2]. β-arrestin binding to the receptor sterically blocks the interaction between GPCR and G proteins, preventing further signaling[3]. β-arrestins also promote endocytosis by functioning as adaptors between endocytotic elements and the receptor[2], promoting internalization of GPCRs through clathrin-coated pits[4]. In addition to receptor desensitization, β-arrestins can coordinate the process of signaling termination through degradation of second messengers[5] such as cAMP[6]. In other instances, arrestins can also act as signaling molecules independent of G-proteins by, for example, binding directly to mitogen activated protein kinases (MAPKs)[3]. Furthermore, upon GPCR activation, β-arrestin1 can translocate into the nucleus, where it facilitates the recruitment of histone acetyltransferase, leading to transcription of genes encoding proteins such as c-fos[7].

Activation of arrestins depends on the phosphorylation of agonist-stimulated GPCRs. Importantly, phosphorylation of GPCRs specifically by GRK enhances the inhibitory effect of arrestins[4]. Furthermore, the pattern of GRK-mediated receptor phosphorylation determines how tightly β-arrestins bind to the activated and phosphorylated receptor[2]. In summary, GPCR activity and signaling are positively and negatively regulated through β-arrestins. Activity of β-arrestins, in turn, depends on the phosphorylation state of the activated GPCR, which is controlled by GRKs.

Overview of G protein-coupled receptor kinases

The seven vertebrate GRKs (GRK1-7) are grouped into three subfamilies: GRK1 (GRK1 and 7), GRK2 (GRK2 and 3), and GRK4 (GRK4, 5 and 6)[8,9]. GRK1 and 7 are exclusively expressed in photoreceptors, where they phosphorylate rhodopsin[10,11]. GRK2 and 3 are broadly distributed in the CNS[12,13], while expression of GRK4 is mostly found in testis[14]. GRK5 is most abundantly expressed in lung, heart, retina, and lingual epithelium, with moderate expression in brain[50,53,54]. The highest expression of GRK6 is found in brain and skeletal muscle, followed by pancreas, and much

lower levels in other organs[15].

All GRKs have in common a multi-domained structure consisting of (1) the N-terminal region, (2) the regulator of G-protein signaling homology domain, (3) a protein kinase domain, and (4) a variable C-terminal domain[16]. In general, the N-terminal region of GRK is believed to recognize the activated form of GPCRs[17]. Lipid modification of the C-terminus, as seen in GRK2, is also involved in this process[18,19]. Binding of a lipid-modified $G_{\beta\gamma}$ subunit to the C-terminus of GRK2 facilitates its translocation from cytoplasm to membrane, where the target GPCR is located. In addition, binding of lipids to the C-terminal pleckstrin homology domain can directly regulate GRK2 activity[20]. In effect, these two mechanisms can synergistically enhance the activity of GRK2[21,22]. Increases in activity of GRKs by lipid binding have been observed for all members of the GRK4 subfamily[23]. In addition to lipid modifications, kinase activity of GRKs can be regulated by other kinases[24]. For example, GRK5 activity can be inhibited by autophosphorylation promoted by calmodulin (CaM)[25-27] or by protein kinase C (PKC)-mediated phosphorylation[28]. Interestingly, GRK2 activity is inhibited by CaM, but this effect is reversed by PKC[29,30]. Other kinases such as Src and MAPK have also been found to have regulatory effects on GRKs[31,32]. In another case, Raf kinase inhibitor protein (RKIP), upon its phosphorylation by PKC, releases from its normal target of Raf1 and binds to GRK2, inhibiting its activity. This change in RKIP function from Raf-1 inhibition to GRK2 inhibition results in enhanced receptor signaling[33]. Taken together, these findings indicate that the activity of GRKs, and therefore subsequent changes in GPCR-mediated signaling, are regulated by many different mechanisms. The effects of CaM and PKC, for example, suggest a model of coordinated regulation of GPCRs[16]. Therefore, any alterations in GRK activity may have a critical impact on cellular functions.

Molecular properties of GRK6

The GRK of most relevance to CNS disorders is GRK6, which is the GRK with highest expression in the brain. Alternative splicing of the C-terminal end of GRK6 yields three variants: GRK6A, B and C, with the sizes of 576, 589, and 560 amino acid residues, respectively[34]. Only GRK6A has the palmitoylation site within the C-terminal domain, which allows membrane localization[35]. The C-terminal region of GRK6B contains consensus phosphorylation sites for PKC and cAMP/cGMP-dependent protein kinases that may contribute to phosphorylation-dependent membrane association. GRK6C, on the other hand, has a truncated C terminus and lacks both the palmitoylation and phosphorylation sites; therefore, it is suspected to have poor membrane association[34,36]. The existence of a fourth variant, GRK6D, has also been reported. This variant, however, lacks a functional catalytic domain and is speculated to act as an inhibitor of other GRK6 isoforms[36].

Palmitoylation of GRK6A has important consequences. Palmitoylated GRK6 is membrane-associated, localized in close proximity to target GPCRs and thus has increased activity[37,38]. However, palmitoylation is not the only mechanism leading to membrane localization for GRK6 variants. When overexpressed in COS-7 cells, the three variants of GRK6 (A, B and C) are membrane-associated despite the lack of putative C-terminal palmitoylation sites in both GRK6B and C[39]. Furthermore, the GRK6C isoform has the highest catalytic activity, suggesting that the C-terminal extensions found in variants A and B, but not in variant C, result in decreased activity[39]. According to the crystal structure, there are two sets of complementary interactions that bring GRK6 into close proximity of a GPCR. First, two regions of GRK6, the α helix region in the N-terminus and the C-terminal tail, directly bind to GPCRs. Second, GRK6 associates with membrane through the phospholipid-binding surface[40]. It has been suggested that the complementary receptor binding provides the free energy needed to induce and stabilize the active form of GRK6[40,41].

While the molecular mechanisms of interactions between GRK6 and GPCRs are becoming better understood, studies attempting to identify the native receptor substrate of GRK6 have yielded inconclusive results. Nevertheless, some candidate receptors have emerged. For example, despite the negative results of earlier studies[42,43], several, more recent studies verified the M3 muscarinic acetylcholine receptor as a target of GRK6[44-46]. Other receptors identified as potential targets of GRK6 are the β2 adrenergic receptor[47] and the insulin-like growth factor 1 receptor[48]. In the CNS, one study demonstrated that the D_2-like dopamine receptor was a target of GRK6[49]. However, more studies are needed to verify the identity of receptor substrates for GRK6 *in vivo*.

GRK6 involvement in PD and animal models of PD

As mentioned earlier, GRK6 is highly expressed in the brain[15]. It reaches its highest expression in the striatum[13,50],

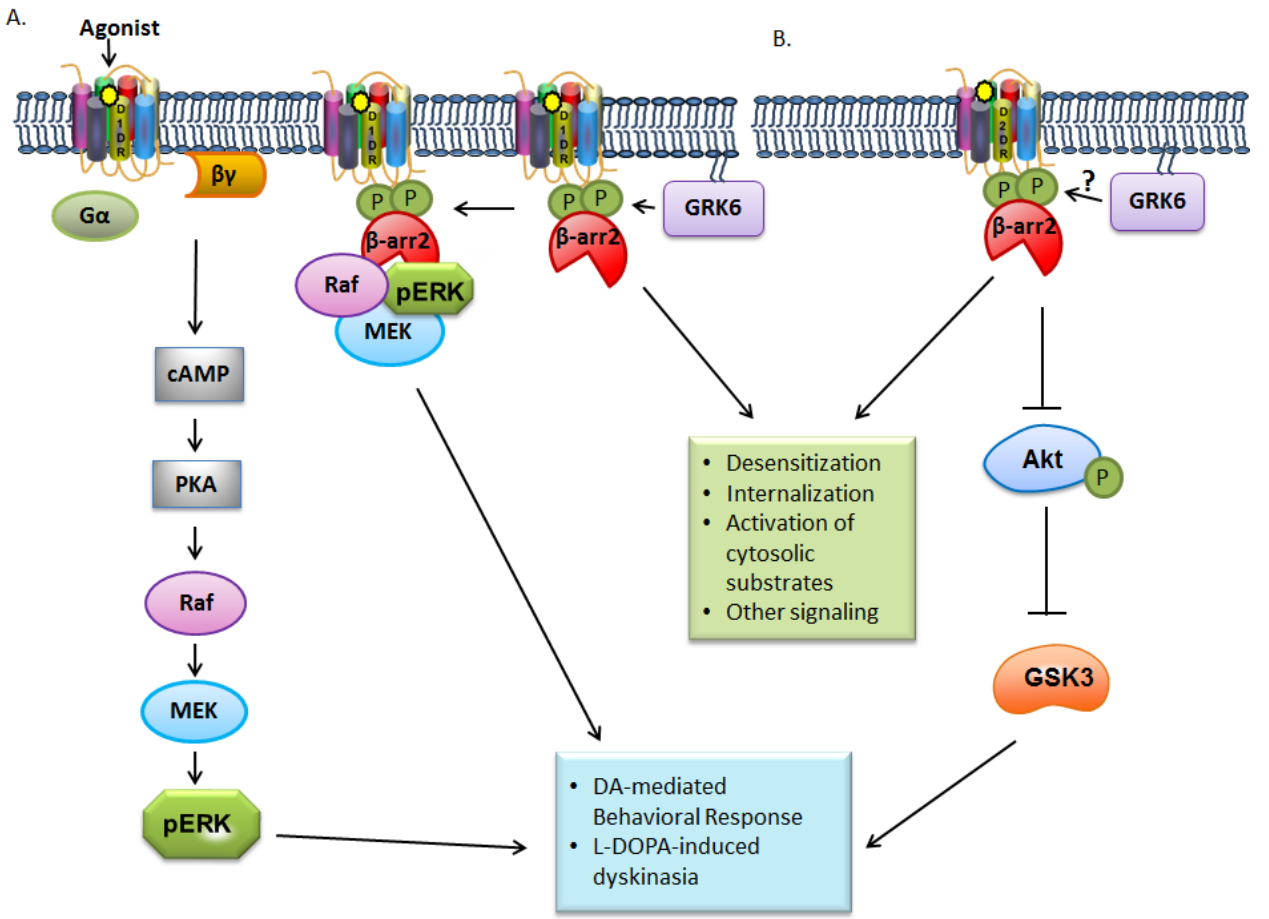

Figure 1: *Signal transduction mediated by dopamine receptors that may lead to Parkinson's disease-related motor dysfunction.* (A) D1 receptor-mediated signal transduction. G protein-mediated signal transduction (left). Activation of trimeric G protein results in increased level of cAMP and activation of PKA, which leads to activation of MAPK cascade and increased Erk phosphorylation. β-arrestin-dependent activation of MAPK (middle). β-arrestin binds to agonist-activated, GRK-phosphorylated receptor, and acts as signal transducer independent of G proteins. Increased activation of ERK by β-arrestin in this manner may contribute to L-DOPA-induced dyskinasia. β-arrestin binding to the activated receptor also leads to receptor internalization (right), followed by receptor desensitization or activation of other signaling cascades. (B) D2 receptor- mediated β-arrestin dependent signal transduction. β-arrestin inactivates Akt through dephosphorylation, and Akt inactivates GSK3 through phosphorylation. Therefore, β-arrestin contributes to dopamine (DA)-mediated signal activation as well as receptor desensitization and internalization. Increased activity of GSK3 has been linked to L-DOPA-induced dyskinasia.

and changes in GRK6 expression levels have been observed in human clinical conditions as well as in animal models of neurological disorders. Therefore, it has been speculated that alterations in GRK6 activity may contribute to pathophysiology of CNS disorders. One of the CNS disorders in which changes in GRK6 have been reported is Parkinson's disease (PD). For example, in postmortem human brain samples, increased GRK6 mRNA levels were detected among the PD patients who also exhibited dementia compared to PD patients without dementia or the control group[50].

PD is a neurological disorder characterized by bradykinesia (slowness of movement), rigidity, and tremor. PD symptoms are the result of a loss of the striatal innervation by dopaminergic neurons in the substantia nigra pars compacta. Currently, L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor of dopamine, is the most effective pharmacological therapy for the treatment of motor symptoms of PD[51]. Unfortunately, after 4-6 years of L-DOPA treatment, about 40% of PD patients develop increased involuntary movements known as dyskinesias. After prolonged L-DOPA therapy, almost 90% of patients develop dyskinesia[52]. The loss of striatal dopamine in PD can be modeled in rodents and non-human primates by injection of dopaminergic neurotoxins. In rats, the neurotoxin 6-hydroxydopamine (6-OHDA) is directly injected into the nigrostriatal pathway or the striatum, whereas dopamine depletion in

non-human primates is achieved by systemic injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Like in humans, dopamine-depleted animals develop dyskinesias following repeated treatment with L-DOPA.

There are a series of studies investigating the role of GRK6 in animal models of PD that were aimed at gaining valuable insight into the molecular mechanisms underlying the altered dopamine signaling in this disorder and, in particular, L-DOPA-induced dyskinesia. Biochemical studies in both MPTP-lesioned monkeys and 6-OHDA-lesioned rats revealed increased GRK6 expression levels in the dorsal and ventral striatum[53,54]. Behavioral studies have also provided insights into the connection between GRK6 and motor function. A study by Ahmed et al. (2010)[55] demonstrated that L-DOPA- or dopamine receptor agonist-induced rotation in 6-OHDA-lesioned rats produces a progression of adverse involuntary movements (AIMs). Similar results were seen in MPTP-lesioned monkeys. It was shown that overexpression of GRK6 alleviated AIMs, and down-regulation exacerbated them. However, in another study, 6-OHDA-lesioned GRK6 kockout (KO) mice showed significantly less dyskinesia as measured by AIM score compared to lesioned wild-type (WT) mice[49]. Therefore, while the former study demonstrated that an increase in GRK6 availability alleviated dyskinesias, the latter study showed GRK6 depletion suppressed dyskinesia. Although these results seem conflicting, it should be noted that there is a caveat in using knockout animals. For example, since GRK2 is also expressed in striatum[54], although at lower levels than GRK6, there may be a compensatory expression of GRK2 or other GRK isoforms in GRK6 KO animals. If that is the case, decreased dyskinesias in GRK6 KO animals may be a result of other GRK isoforms being upregulated. Further studies are needed to address these possibilities.

To further understand the role of GRK6 in dopamine receptor-mediated locomotor activity, Gainetdinov et al. (2003)[56], using GRK6 KO mice, took a pharmacological approach in the GRK6 KO mice. This study showed that GRK6 KO mice had an increased locomotor response to the D_2 agonist quinpirole. *In vitro*, GRK6-mediated desensitization of D_2-like, but not D_1-like dopamine receptors. In mouse striatum, one possible signaling mechanism downstream of the D_2 dopamine receptor in which GRK6 plays a role involves Akt and glycogen synthase kinase 3 (GSK3)[57]. Activation of β-arrestin 2 via D_2 receptor stimulation leads to dephosphorylation and inactivation of Akt which, through phosphorylation, has an inhibitory effect on GSK3. In support of GRK6's involvement in this signaling cascade, GRK6 KO mice had significantly higher basal levels of pGSK3β compared to WT mice[49].Interestingly, there are also some data suggesting that GRK6 may exert its effect on dyskinesia via the MAPK signaling cascade. For example, after continuing L-DOPA treatment on 6-OHDA-lesioned mice, expression of phosphorylated extracellular signal-regulated kinase 2 (pErk2), one member of the MAPK family, was significantly increased compared to sham-operated control. There were no changes in pErk expression, however, between controls and 6-OHDA-lesioned GRK6-KO mice[49]. In a separate study, Westin et al. (2007)[58] showed that there was a positive correlation between the severity of L-DOPA-induced dyskinesia and the amount of pErk expression in 6-OHDA-lesioned rat striatum. In addition, in those animals, application of a D_1 antagonist reduced the development of dyskinesia as well as the amount of pErk. Together, these results suggest that dyskinesia induced by the action of L-DOPA on D_1 receptors may involve GRK6 through its activation of the MAPK cascade.

GRK6 as a drug target

Development of drugs for CNS disorders has been less successful than in other areas of disorders because of limited knowledge of the underlying pathophysiology, limitations in current preclinical models of CNS disorders, and a lack of good biomarkers[59]. For treatment of PD, there are many available drugs that include, for example, L-DOPA, D_2 dopamine receptor agonists, catechol-O-methyltransferase inhibitors, and monoamine oxidase inhibitors. Despite the fact that L-DOPA treatment loses efficacy with time and produces adverse side effects, it remains the most widely used therapy today due to the limited efficacy of other drugs[60]. Currently, amantadine, a non-competitive N-methyl-D-aspartate receptor antagonist is the only drug used to alleviate the symptoms of L-DOPA-induced dyskinesia, but prolonged use of amantadine leads to the development of adverse side effects such as dizziness, confusion and hallucinations[51]. Another, non-pharmacological approach to symptomatic treatment of PD that can reduce L-DOPA induced dyskinesia is deep brain stimulation (DBS) of the subthalamic nucleus. DBS, however, involves neurosurgeries that are expensive, come with potential risks, and require regular maintenance of the stimulator[51]. Because of the limitations associated with all the current therapies, there is a need for continuing efforts to develop novel therapies aimed at new potential drug targets. As an

alternative to drugs that directly activate or inhibit GPCRs, there is the potential for therapeutic value in regulators of GPCR activities[1]. Although lagging behind, continuing efforts have been made for development of drugs targeting CNS protein kinases such as GSK3 for Alzheimer's disease and neuropsychiatric disorders, PKC for bipolar disorder, and mammalian target of rapamycin for autism[61]. Some interesting properties of GRK-mediated GPCR regulation make GRK an attractive drug target. Probably one of the most intriguing discoveries is the ability of GRK-produced phosphorylation patterns to stimulate distinct downstream signaling through β-arrestin in a GRK variant-specific manner[62]. The mechanism of this process was unlocked in a study by Nobles et al. (2011)[63]. In this experiment, the authors demonstrated that, although both GRK2 and GRK6 participated in the phosphorylation of the β_2 adrenoreceptor, the functional outcome was different depending on the distinct phosphorylation patterns that were produced by each kinase. Furthermore, binding of different ligands to GPCRs could determine which GRK would be activated.

Conclusions and future directions

As discussed in the previous sections, several lines of evidence suggest that GRK6 has a critical role in PD. Its tissue-specific expression and intricate network of regulatory functions make GRK6 a good drug target for PD treatment. The future of successful drug development may lie in the discovery of selective ligands for GRK6, which may enable precisely targeted treatment for CNS disorders and/or alleviate undesired side effects when used in combination with other GPCR ligands. By manipulating the activity of GRK6, it is possible to selectively target and fine-tune the cellular signaling events that contribute to CNS disease phenotypes downstream of GPCRs.

One of the most prominent drug-discovery techniques, high-throughput screening (HTS), is a promising route to identifying possible GRK6-targeted drugs. HTS of compound libraries has evolved into a successful approach for discovering novel drugs for both CNS disorders and other indications. Examples of successful HTS efforts include the discovery of drugs that target tyrosine kinase for cancer treatment (e.g. Gefitinib, Lapatinib), proteases for HIV (Tipranavir) and GPCRs for hypertension (Ambrisentan)[64]. It should be noted, however, that screening for drugs that target protein kinases is in its infancy, and there are undoubtedly many challenges in the process. Some of the difficulties include assay design, finding compounds with sufficient affinity and selectivity for the target as well as appropriate chemical properties for CNS penetrance[61]. Another inherent challenge is that most protein kinase inhibitors identified by HTS are ATP derivatives that compete for the binding site with endogenous ATP[65]. Therefore, it will require careful planning and optimization of the assay conditions before screening attempts should be made. With utilization of proper conditions and technique, the HTS approach may lead to the discovery of compounds that selectively act on GRK6 and could be used to further define the role of GRK6 in PD.

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CANDIDATE REVIEWS

Using Context to Search Memory: Functional Roles of the Medial Temporal Lobe and Prefrontal Cortex

James Kragel

The ability to search one's past for a specific memory amongst similar occurrences is a hallmark feature of the episodic memory system. Context-based theories of episodic memory retrieval propose that a slowly changing contextual representation allows for the construction of discrete, separable episodes. A review of recent neuroimaging, neuropsychological, and electrophysiological evidence suggests that activity within the medial temporal lobe (MTL) may support such a contextual representation, while control processes within the prefrontal cortex may influence the maintenance and updating of representations within the MTL.

Keywords: *Episodic memory, medial temporal lobe, prefrontal cortex*

Episodic memory: Memory of events, incorporating contextual information such as the time, place, and emotional state of the memory.

Item recognition: A memory task in which participants must determine whether a stimulus has been presented previously.

Source memory: Memory for contextual details associated with a particular episode.

Introduction

Cognitive theories of episodic memory posit that memory retrieval involves patterns of neural activity returning to a previous state[1, 2], resulting in the subjective experience of 'mental time travel'[3]. These cognitive models rely on a contextual representation that integrates information over long time scales, representing information about both the environment and internal states. When an item is encoded into memory, associations are formed between the item representation and the current state of context. This associative link allows for two different processes to occur: 1) a state of context can be used to retrieve particular items from memory; and 2) particular items can be used to retrieve contextual information, such as the location or time in which they were studied. When an item is retrieved, the context in which it was studied may also be retrieved, causing the contextual representation to return to its prior state. A growing body of evidence supporting the representation of item information within posterior cortical regions, as well as their reactivation during memory retrieval, will be reviewed. While there is ample support for the neural representation of item-specific information, only recent investigations have begun to search for neural mechanisms that may mediate a contextual representation. Findings from the neuroimaging and neuropsychological literature that support the role of MTL and prefrontal regions in the processing of contextual information will be reviewed.

Reactivation of cortical activity during retrieval

Recent neuroimaging studies provide evidence that retrieval of a previously bound episode occurs when the cortical activity present when the memory is constructed is reactivated. Studies examining cortical reactivation typically have similar experimental form, in which a particular cue is presented during the encoding of an episodic memory. Neural recording during retrieval, when participants typically perform an item recognition or source memory task associated with a specific cue, has provided evidence that cortical activity related to modality (auditory or visual)[4, 5], encoding task (imagine or read)[6], or type of odor[7] reactivates during these retrieval tasks. Critically, the reactivation of contextual details during these tests is driven by associative retrieval mechanisms, as only the cue is presented during test. Studies of this nature have shown the reactivation in visual and auditory cortex during the associative retrieval of visual[4, 8-11] and auditory information[4, 5]. These findings of reactivation in sensory association cortex are supportive

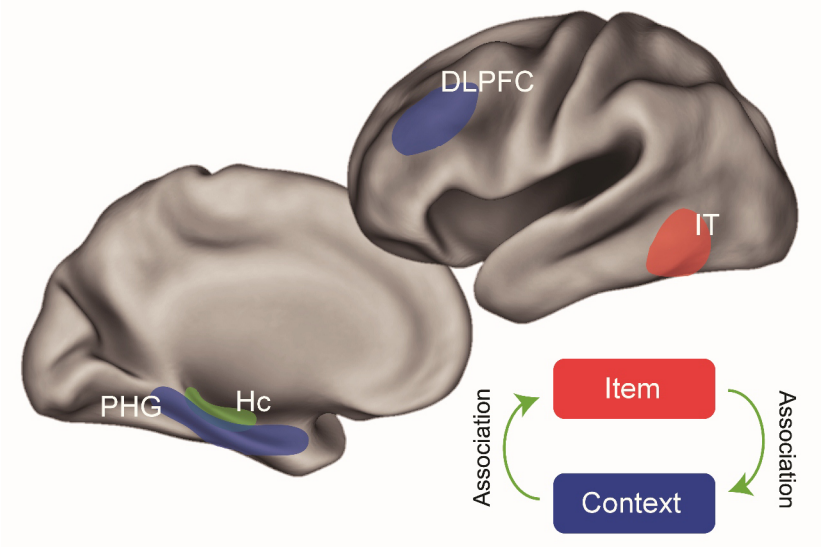

Figure 1: *Schematic diagram mapping cortical regions implicated in maintaining and updating context (blue), representing the features of items (red), and forming associations between items and the context in which they are encountered (green).* PHG, parahippocampal gyrus. Hc, hippocampus. DLPFC, dorsolateral prefrontal cortex. IT, inferior temporal gyrus.

of context-based theories of episodic memory, which rely on a representation of item information that activates when a specific item is presented during study, or retrieved through mnemonic processing.

In addition to the activation of associated perceptual information, studies have revealed that activity specific to the type of processes engaged during encoding may also be reactivated during retrieval. Work by Johnson and Rugg[12] demonstrated cortical reactivation in left occipital cortex and anterior fusiform gyrus when participants imagined a verbally presented object in a visual scene, while activity in ventromedial prefrontal cortex was reinstated when the same stimulus was incorporated into a sentence. Work by Kahn and colleagues[6] showed cortical reactivation in the parahippocampal place area (implicated in processing scene information[13]) for imagined items, while items that were read reactivated posterior ventrolateral prefrontal cortex (implicated in lexical tasks). These findings suggest that contextual information beyond sensory perception is bound into episodic memories.

The above neuroimaging studies rely upon the presentation of a cue during test in order to test for the associative retrieval of contextual details. Context-based theories of episodic memory propose that retrieval may occur in the absence of an external cue, as memory search is guided by an internally maintained contextual cue. In support of the contextual reinstatement hypothesis, Polyn and colleagues[14] found cortical reactivation in the visual processing stream during a free recall task. In this study, fMRI data was collected while participants studied images of famous celebrities, locations, or objects, and later retrieved them during a free-recall task. Using multivariate pattern analysis techniques, activity in fusiform gyrus, parahippocampal cortex, and inferior temporal gyrus was shown to reactivate prior to the recall of celebrities, locations, and objects, respectively. These findings are consistent with category-specific activity cuing memory retrieval. In line with the reactivation of task-specific activity in source memory studies, reactivation of task specific activity in occipital cortex, parietal cortex, and lingual gyrus was observed during a similar free-recall task[15].

Perhaps the most compelling evidence of reactivation of neural activity, driven through the hippocampus, comes from electrophysiological recordings of hippocampal neurons and their surrounding local field potentials from patients with intractable epilepsy. Gelbard-Sagiv and colleagues[16] recorded activity in single neurons of the human hippocampus while patients first viewed cinematic episodes, and later recalled these episodes. A subset of recorded neurons exhibited selective firing throughout

Free-recall task:
A memory paradigm in which participants study a list of items on each trial, and are prompted to recall the items in any order, without a retrieval cue.

Oscillatory activity: Synchronized activity of a large number of neurons firing at a specific frequency.

Hebbian learning: An associative learning process in which connected neurons that exhibit simultaneous firing strengthen their synaptic connections to one another.

the presentation of the encoded materials. Reactivation of these neurons preceded recall of the encoded material, directly linking hippocampal firing and human recollection during memory search. Reactivation of oscillatory activity in the gamma band has also been observed during successful encoding during a free-recall paradigm[17]. This activity, localized to prefrontal, hippocampal, and left temporal lobe electrodes was shown to reactivate during the recall of studied items. Reactivation occurred first within the hippocampus, before the pattern of activity in cortex was reinstated. Findings of this nature are suggestive of recall of past experience originating in the MTL, and propagating to association cortex, where activity reflects the subjective experience of the recollected memory.

Bridging cognitive models and neural mechanisms

In the following section, evidence for the neural substrates mediating the major cognitive processes proposed by context-based theories will be briefly reviewed. These theories propose that activity in neocortical association cortex reflects information about individual items recently encountered in the environment. In addition, a context representation contains information about the recent history of items that have been active in association cortex; these theories propose this activity is represented in either prefrontal[18] or parahippocampal[19] regions. Finally, these theories propose that the hippocampus mediates the binding of item and context representations, in the formation of distinct episodes that may be later retrieved. The recall of a specific item, coincident with cortical reactivation of the pattern of neural activity representing said item, is enacted by similar hippocampally mediated Hebbian learning. In this manner, these models provide a means to explain the phenomenon of cortical reactivation during episodic remembering (depicted in **Figure 1**).

Extant neuroimaging evidence speaks to the role of the hippocampus in the successful encoding[12, 20-23] and retrieval [23-26] of episodic memories. There is also a consensus in terms of object representations in association cortex based on modality: superior temporal gyrus for auditory representations, and inferior temporal gyrus for visual representations. Within the ventral visual pathway, specific cortical regions encode specific stimuli, such as the fusiform face area[27], the parahippocampal place area[13], and object responsive areas in lateral occipital cortex[28]. Given the recent advent of context-based theories of episodic memory, limited but promising progress has been made towards identifying potential substrates that mediate a contextual representation. In the following section, evidence supporting parahippocampal cortex activity mediating a temporal context representation will be reviewed. Evidence for the role of the dorsolateral prefrontal cortex (DLPFC) in manipulating, or shaping the information stored in this representation will also be considered.

Contextual maintenance and integration

The existence of a slowly changing contextual representation is essential to models of episodic memory. According to the temporal context model[1], changes in context are driven by perceptual inputs to the system, with the similarity of context to prior states decaying in an exponential manner with additional inputs. In order for a region to represent context, patterns of neuronal firing must be capable of changing firing rates based on external inputs (proposed here to reflect perceptual inputs due to feature information), as well as maintain persistent firing over delay periods. Persistent firing enables the maintenance of information in the absence of external stimuli, a critical property of context representations. Persistent firing of cortical assemblies has been recorded in prefrontal cortex[29, 30], limbic regions, including entorhinal cortex[31, 32], perirhinal cortex[33, 34], and lateral amygdala[35] of nonhuman primates and rodents (for a review, see work by Wang[36]).

Parahippocampal cortex

Given the firing patterns observed in parahippocampal regions, their afferent sensory inputs, and extensive reciprocal connections to the hippocampus, activity in parahippocampal cortex is well suited to mediate a contextual representation[37, 38]. Studies examining working memory in humans have identified a dissociation between the maintenance of novel information, supported through MTL function, in contrast to familiar stimuli, supported in part by increased prefrontal activation[39]. Similar work has identified that successful encoding of novel visual information is supported by the parahippocampal gyrus[40], which can be reduced through the application of the muscarinic cholinergic antagonist, scopolamine, prior to scanning[41]. Loss of cholinergic modulation, and consequently recurrent firing, to entorhinal cortex also produces reduced encoding of novel, but not familiar odors, in rats[42].

The most compelling evidence for a temporal context representation supported through activity within the MTL comes from recent electrophysiological studies recording from the MTL in humans. Work by Manning and colleagues[43] found direct support for contextual reinstatement, evidenced by the reactivation of oscillatory patterns of brain activity recorded in local field potentials, while patients with intractable epilepsy studied a list of nouns and performed free recall. In their analysis, they identified a slowly changing, autocorrelated signal while patients studied specific items- a contextual representation. Critically, just prior to the recall of individual items, the pattern of contextual activity recorded from temporal lobe electrodes, including the hippocampus, returned to a prior state. Recent recordings from within the MTL have also revealed autocorrelated patterns of neuronal firing, which return to a previous state of firing during a continuous recognition task[44]. These studies build on a growing body of evidence from rodent[45, 46], nonhuman primates[47], and humans[48, 49] for the encoding and maintenance of temporal context within the MTL.

Dorsolateral prefrontal cortex

The DLPFC has been proposed to act as central locus for a contextual representation[18, 50], due to its sustained delay period activity in working memory tasks, as well as deficits in source memory[51-53], and recency judgments[54, 55] with frontal lesion pathology (although explicit contextual encoding remains intact in frontal amnesics[56, 57]). Furthermore, patients with frontal lobe lesions have been shown to have spared automatic encoding of temporal information, but deficits in the processing of this information[58]. Functional neuroimaging evidence also points to the role of DLPFC in the selection of action from memory[59, 60] rather than maintenance of information (reviewed by Curtis and D'Esposito[61]). Recent evidence from neural decoding of a prospective memory tasks supports the role of prefrontal cortex in modifying the contents of working memory, which could be decoded in posterior cortical regions (but not task-sensitive regions in anterior and lateral prefrontal cortex)[62]. This top-down modulation of contextual representations fits in line with the temporal patterns of firing in electrophysiological recordings of DLPFC[63], as well as lesions studies of DLPFC and inferior temporal cortex[64]. This evidence speaks to an interactive role of the DLPFC, with a potential role in the organization of information stored in context.

Recent neuroimaging work investigating the role of DLPFC provides evidence for its role in organizing currently active representations held in working memory[65]. In this study, participants encoded a list of three items, and either performed rote rehearsal or reordered the items according to their weight, during a delay period. This reordering task requires additional manipulation of the contents of working memory, as compared to the rehearsal control task. Consistent with the theorized role of DLPFC in the organization of currently maintained representations, activity in DLPFC was greater for reordering, relative to rehearsal, of items in working memory. Increased activity within DLPFC has also been linked to subsequent relational memory effects[66, 67], and subsequent clustering[68] and memory[69] of studied materials during free recall. These findings support a role for the DLPFC in the encoding of associations between currently active item representations; activity in this region may directly interact with inputs to a contextual representation, allowing individual items presented across longer time scales to be associate to similar contextual states. Future studies investigating how activity within DLPFC during working memory tasks influences item representation in posterior cortical regions may provide further insight into the functional role of the DLPFC.

In addition to supporting relational encoding of items, neuroimaging studies of lateral prefrontal cortex function suggest it may mediate temporal context encoding. As previously mentioned, lesions to prefrontal cortex often cause deficits in recency discrimination[54, 70-72]. Motivated by evidence from the neuropsychological domain, Jenkins and

Ranganath[48] examined prefrontal and MTL contributions to the encoding of temporal-order memory. In this study, participants encoded a series of four stimuli, and after an eight second delay, were required to indicate from which position a test probe was encoded in the list. Activity during trials in which subjects correctly assessed the position of the probe was compared to activity during incorrect trials. This contrast of fine temporal memory encoding revealed activity in parahippocampal cortex. In addition to this within-trial measure of temporal context encoding, participants were given a post-scan test in which they indicated the temporal position an item was presented in on a time line representing the duration of the experiment. Activity in rostrolateral prefrontal cortex, DLPFC, and anterior hippocampus promoted the encoding of coarse temporal memory. To test if the pattern of activity in any of these regions represented a gradually changing contextual representation, a multivariate pattern analysis of activity in regions showing temporal memory effects was conducted. In this analysis, patterns of activity were constructed in each region of interest, and the similarity of these patterns was compared to adjacent trials. Trials with accurate coarse memory were associated with a dissimilar pattern of activity, relative to neighboring trials. If this pattern of activity in fact represents the encoding of temporal context, the distinct temporal context may facilitate subsequent temporal order judgments. Alternatively, if items are encoded with similar contextual states, it may be more difficult to distinguish when they occured, relative to other items in the list. These findings, as well as other reports of DLPFC activity supporting subsequent temporal order memory in similar studies[49] supports the role of prefrontal processes in supporting the encoding of temporal context.

Conclusion

Recent findings suggest that activity in the MTL may mediate a contextual representation, critical for episodic memory. The manner in which information is gated into the medial temporal lobe may be controlled by activity in prefrontal frontal cortex, allowing for control over the current contextual representation. Future studies should emphasize how interactions between prefrontal cortex and the medial temporal lobe shape the current state of context.

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Modeling Gene-Environment Interactions in Parkinson's Disease Using Patient-Derived Induced Pluripotent Stem Cells

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Parkinson's disease (PD) is a highly prevalent, progressive neurodegenerative disease characterized by loss of the dopaminergic neurons of the substantia nigra (SN) pars compacta. Although there are several proposed mechanisms for the pathophysiology of this debilitating illness, efforts to develop disease-modifying therapies have been hampered by the inability of existing model systems to completely reproduce the characteristic molecular and pathological features of PD. Given the potential of both environmental toxicants and genetic risk factors to modulate the onset and severity of PD, a model system that accounts for both would serve as a valuable tool in the study of PD-related environmental neurotoxicants. The advent of induced pluripotent stem cell (iPSC) technology has created the opportunity to evaluate personalized, toxicological susceptibility to specific environmental agents. Using this system, it is now possible to analyze cellular physiological pathways in human neurons, both developing and mature, and glial cells that play key roles in handling neurotoxicants. Furthermore, the utilization of living human cells with identical genetic determinants as the resource subjects, with or without PD, is a powerful resource for the development of therapeutics that modulate patient susceptibility to environmental toxicants.

Keywords: *Parkinson's disease, manganese, induced pluripotent stem cells*

Epidemiology of PD

Parkinson's disease (PD) is the second most common neurodegenerative disease. The prevalence of PD in the industrialized world is estimated at 0.3% of the general population and approximately 1% in individuals over the age of 60[1]. Thus, PD is considered an age-related disease, with prevalence rising mainly after the age of 50[2-12]. As the population ages, there is an increasing socioeconomic burden on society[2]. The incidence of PD is 8 to 18 per 100,000 person-years[2]. It has been noted that there is higher prevalence of PD in men than women, a finding hypothesized to be mediated by a neuroprotective role of estrogens[2, 3, 6-8].

PD diagnosis is contingent on presentation with at least two of the four cardinal symptoms: resting tremor, bradykinesia, rigidity, and postural instability[2]. The clinical suspicion is further supported by the patients' responsiveness to levodopa, asymmetry of symptoms, or SPECT imaging with DaTSCAN, although the latter is seldom used as a primary diagnostic procedure[13, 14]. Furthermore, secondary causes of parkinsonism, such as drug-induced parkinsonism, must be excluded. Interestingly, the course of PD is highly variable; studies analyzing PD progression suggest that functional deterioration is accelerated both early in the disease course and among patients presenting with postural instability gait difficulty[15-18]. The majority (90%) of PD cases are sporadic in etiology, with the remaining 10% of cases having known genetic causes. Furthermore, there is profound heterogeneity in age of onset, neuropathological findings, and characteristic symptoms even among the genetic forms of PD.

Pathophysiology of PD

Although a complete understanding of the

Bradykinsia:
A slowness in the execution of movement. It is one of the three key symptoms of parkinsonism, which are bradykinesia, tremor and rigidity.

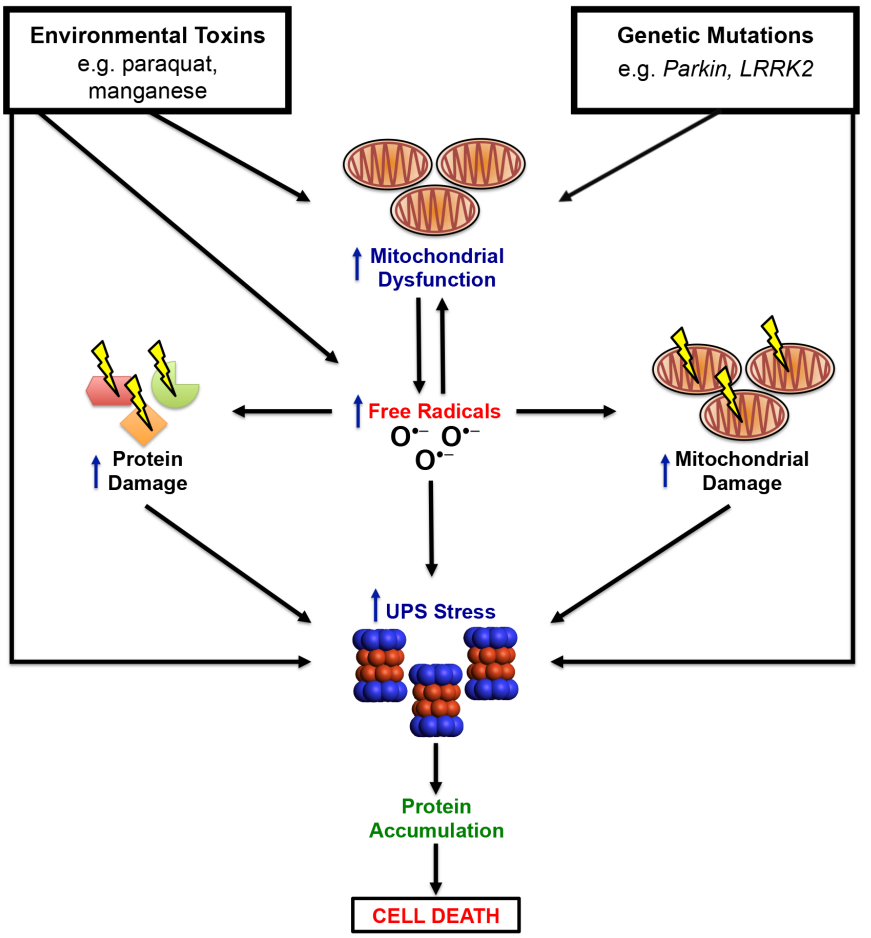

Figure 1: *Gene-environment interactions in PD.* Environmental and genetic factors result in the mitochondrial dysfunction, generation of free radicals, mitochondrial/protein damage, increased UPS stress, protein aggregation, and dopaminergic cell death in PD.

Ubiquitin proteasome system (UPS): A multicomponent system that identifies and degrades unwanted proteins in the cytoplasm of all cells; involved in cell growth and differentiation, DNA replication and repair, apoptosis, and stress and immune responses.

pathogenesis of PD remains elusive, current evidence suggests that PD results from a multitude of factors, including: oxidative stress, protein aggregation, and mitochondrial dysfunction[19]. PD is characterized by loss of dopaminergic neurons of the substantia nigra and the presence of intraneuronal α-synuclein protein aggregates composed of α-synuclein known as Lewy bodies[20]. Loss of dopamine levels in the striatum leads to downstream dysregulation of basal ganglia motor circuitry, resulting in the motor symptoms observed in PD. Studies exploring the genetic forms of PD have offered insight regarding central mechanisms in PD pathogenesis. Defective proteins in familial PD result from mutations in genes that function in critical cellular processes, such as the ubiquitin proteasome system (UPS), vesicle trafficking, mitochondrial function, and oxidative stress responses[21-23]. These findings suggest that while there is a common endpoint of decreased striatal dopamine levels, multiple pathways can influence an individual's pattern of neuronal cell death and the mechanism by which it occurs. For example, dysfunction in mitochondrial complex I results in upregulated free radical production causing protein damage. The damaged protein burden increases the stress on the UPS, leading to protein aggregation and subsequent neuronal death[24]. However, this simple pathway could be influenced at any level by multiple inputs, such as environmental toxins, genetic risk, and enhanced oxidative stress **(Figure 1)**. Thus, an individual patient's history of environmental exposure and genetic risk are critical to their clinical manifestation of PD.

Dystonia: A neurological movement disorder, in which sustained muscle contractions cause twisting and repetitive movements or abnormal postures.

Environmental Influences in PD

Epidemiological and laboratory research has revealed a number of environmental exposures and toxicants that contribute to PD. One of the most notable of PD-causing environmental toxicants is 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), a contaminant of a synthetic heroin analogue, which was identified as the culprit in the dramatic onset of parkinsonian symptoms in four individuals after self-administration of the drug[25]. MPTP administration to both mice and non-human primates revealed a buildup of α-synuclein within nigral dopaminergic cell bodies[26, 27]. These MPTP studies produced both a valuable model system of parkinsonism and launched a wave of investigation focusing on environmental exposures that contribute to PD.

Environmental Exposures: Pesticides, Heavy Metals, and Beyond

One of the most well-studied environmental associations with PD is that of pesticides and herbicides. Case control studies have revealed an association between exposure to herbicides, insecticides, and farming as an occupation and PD[28, 29]. Individuals exposed to pesticides have a 70% higher incidence of PD compared to those not exposed[30]. The timing of exposure has a profound impact on susceptibility; exposure at a younger age increases PD risk relative to those exposed over the age of 60[31]. However, these studies are limited to self-reporting by subjects; , a variable that is highly dependent on the awareness of the individual and subject to recall bias. In addition, subjects recruited for these studies would most likely be unable to report prenatal or early-childhood exposures, further limiting the interpretation of epidemiological data. Despite these limitations, several major pesticides have been associated with PD, including: dieldrin, maneb, paraquat and rotenone[32, 33]. Although it is outside the scope of this article to discuss the mechanism of these toxicants individually, paraquat is representative of how such exposures produce neurodegeneration resulting in parkinsonism, and will be given a brief discussion here.

The toxic properties of paraquat are caused by its redox cycle in which it is reduced by NAPDH-CYP450 reductase, NAPDH-cytochrome *c* reductase, and mitochondrial complex I[34-37]. This process generates a paraquat monocation free radical that is rapidly re-oxidized, producing the superoxide radical (O_2^{2-}). This process sets off a cascade of reactions in which more reactive oxygen species are generated, leading to cellular stress and, when the cellular antioxidant capacity is overwhelmed, eventual degeneration. Furthermore, paraquat is known to generate selective neurodegeneration of SN dopaminergic neurons when administered to rodents in a manner similar to that of MPTP.[38] In addition to its acute effects, paraquat exposure during critical developmental periods generates progressive and permanent lesions of the SN dopaminergic system, rendering it hypersusceptible to adult neurotoxicant exposures[39].

In addition to pesticides, exposure to heavy metals is associated with PD. In particular, exposure to iron, manganese, lead, copper, zinc, amalgam, and aluminum have each been demonstrated to increase risk of PD[40]. This is of direct clinical relevance, as studies have shown that high manganese exposure produces a secondary form of parkinsonism, known as manganism, that is clinically indistinguishable from idiopathic PD aside from age of onset.[41]. The mechanism of neurodegeneration in heavy metal toxicity is hypothesized to be due to deposition of the metals in the SN and increased oxidative stress.[40].

Important to note, not all environmental exposures are neurotoxic, several agents have a demonstrated neuroprotective role such as coffee drinking, smoking, and statin use.[42, 43]. This suggests that environmental impact on PD is bidirectional, and recommendations regarding neuroprotective strategies may be of utility in those at high risk for developing the disease.

Rasagiline:
An irrever-sible inhibitor of monoamine oxidase used as a monotherapy in early Parkinson's disease or as an adjunct therapy in more advanced cases.

Genetic Influences in PD

Several loci and genes have been identified as causative of the genetic forms of PD. These include the autosomal dominant *PARK1* and *PARK4* (*SNCA*/α-synuclein), *PARK5* (*UCHL1*), *PARK8* (*LRRK2*), *PARK11* (*GIGYF2*), *PARK13* (*Omi/Htra2*) and the autosomal recessive *PARK2* (*Parkin*), *PARK6* (*PINK1*), *PARK7* (*DJ-1*), and *PARK9* (*ATP13A2*)[44]. These genetic forms vary significantly between each other and are different from the sporadic forms of PD in their age of onset, clinical course, and response to treatment. One example of how a genetic mutation can produce PD is illustrated by *PARK2* loss-of-function mutations, the most common cause of autosomal recessive juvenile parkinsonism (ARJP). In addition to the cardinal symptoms of PD, ARJP is distinguished by prominent lower limb dystonia, severe levodopa-induced dyskinesias, and early age of onset, usually before the age of 40[45]. Notably, patients with ARJP caused by a mutation in *PARK2,* have loss of dopaminergic neurons without the presence of Lewy bodies[20]. Parkin (the protein product of *PARK2*) functions as an E2-dependent E3 ubiquitin ligase that functions as a substrate-recognition molecule within the UPS[46]. It is hypothesized that loss of Parkin function of Parkin results in reduced ubiquitination of its substrates and subsequent protein accumulation and toxicity to dopaminergic neurons (**Figure 1**)[47, 48]. There is evidence that Parkin plays a critical role in engulfment of mitochondria with low membrane potential, leading to the hypothesis that failure to eliminate dysfunctional mitochondria contributes to PD pathogenesis.[49]

In addition to these monogenic forms, sporadic forms of PD are likely influenced by an individual's global genetic variation. A number of genome-wide association studies (GWAS) have identified particular PD-associated loci. Susceptibility loci include regions within the monogenic causative genes such as SNCA (4q22) and LRRK2 (12q12), as well as newly identified loci such as PARK16 (1q32), BST1 (4p15), and *HLA-DRA*[50-53]. Given that the previously discussed environmental toxicants act on related processes, genetic findings have generated interest in the study of gene-environment interactions that may underlie the heterogeneity in presentation among PD patients.

Gene-environment interactions in PD – Modeling neurotoxicological risk

Examination of neurotoxicological risk in PD model systems has emerged as an area of active research given the strong evidence for the influence of both environmental and genetic factors on PD onset. The concept of mutations in individual genes or single nucleotide polymorphisms across multiple loci that alter the susceptibility of an individual to a given toxicant has been validated in many studies. For example, mutations in the gene *MDR1* predispose individuals to the injurious effects of pesticides and other P-glycoprotein transported xenobiotics, resulting in PD[54]. Similarly, polymorphisms in metabolic enzymes such as MAOB, CYP2D6, and GSTT1 have been associated with PD[55]. On the other hand, experiments in yeast and animal model systems have revealed a protective role of certain genes, such as *PARK9* (ATP13A2) that protects against manganese toxicity and dopaminergic cell death due to α-synuclein overexpression[56]. Despite these advances, there is a knowledge gap between clinical data from PD patients and laboratory data generated using model systems. It is assumed, however, that by modulating individual response to neurotoxicants, the clinical course of PD can be manipulated. This concept has been demonstrated in clinical trials demonstrating the beneficial effects of levodopa, tai chi, and rasagiline on PD symptoms and progression.[57-59]. Thus, the need to test different interactions between subject-specific genetic background and environmental exposures makes patient-specific iPSCs a powerful tool to predict clinical outcomes and guide clinical investigations and intervention.

The utility of iPSC technology for neurotoxicology

Background of iPSCs

In 2007, Takahashi *et al.* reported for the first time the possibility of reprogramming adult human-derived fibroblasts to pluripotent stem cells using four defined transcription factors, *OCT4*, *SOX2*, *c-MYC*, and *KLF4*[60]. iPSCs exhibit the typical characteristic of inner cell mass-derived human embryonic stem cells, including self-renewal and the ability to differentiate into cell types of all three germ layers. This landmark study launched a new field of research focused on improving the efficiency of reprogramming and deriving cells from various patient types. Initial experiments utilized retroviruses for transduction, which introduced a set of drawbacks including mutagenesis at insertion sites and persistent expression of reprogramming factors. In efforts to overcome these obstacles, several alternative reprogramming strategies have been developed, including doxycycline-inducible expression, the use of *loxP* sites, *PiggyBac* transposons, adenovirus transduction, plasmid transfections, and episomal vectors[61-67]. In addition, other groups are investigating the use of compounds that permit iPSC induction without the use of genetic material[68]. These improvements in reprogramming enhance the utility of this system for the study of gene-environment interactions, as they minimize the contribution of the reprogramming process to genetic heterogeneity among iPSCs derived from different individuals. After reprogramming patient fibroblasts to iPSCs, the cells can be differentiated into a variety of neuronal and glial subtypes including functional midbrain dopaminergic neurons[69, 70].

iPSCs as a model for gene-environment interactions in PD

One of the major advantages of utilizing patient-specific iPSCs to study neurotoxicological interaction is that an individual may be evaluated for environmental risk without *a priori* knowledge of their genetic risk factors[71]. The use of iPSCs for toxicological risk assessment is dependent on the assumption that cells derived from patients serve as a model system for understanding the influence of human genetic factors and their ability to modulate the vulnerability of differentiated cells to a given toxicant[71]. Although efforts to validate these assumptions are underway, iPSC technology remains an exciting opportunity to examine changes in the development and maintenance of neuronal function after genetic and toxicant perturbation.

There are a variety of exposure paradigms where iPSC-derived neurons and neural progenitors are of value. Through this experimental system, environmental insults or protectants can be screened across different temporal delivery patterns to understand response to acute and chronic exposures. The pattern of exposure is of interest, since toxicants such as methylmercury exhibit acute and latent effects with variable sensitivity based on developmental time point[72-74]. *In vitro* neuronal differentiation of hiPSCs permits assessment of interactions between early exposure and subsequent risk of neurodegenerative phenotypes in acute, multi-hit, and chronic exposure paradigms. Prior to iPSCs, such studies could only be performed utilizing primary cell culture or embryonic stem cells, which are high-cost alternatives that lack patient specificity.

Furthermore, developing neural progenitors can be exposed to chronic low concentrations of the agent to mimic the effect of cumulative toxicity across the lifetime of a neuron. The pluripotent nature of iPSCs allows the assessment of a diverse set of neuronal subtypes to a given exposure. For example, one could investigate if midbrain dopaminergic neurons have a heightened susceptibility to manganese during development compared to forebrain dopaminergic neurons derived from the same patient. Alternatively, polymorphisms at different loci between patients could heighten developmental sensitivity to a toxicant between PD patients and controls. Such findings from iPSCs can inform downstream *in vivo* vertebrate studies that account for endogenous processes such as detoxification, neuronal regeneration, and immune response.

Current challenges in modeling PD with iPSCs

Perhaps the greatest challenge utilizing iPSCs is assuring the coherence of genotype and phenotype. Many groups have identified methylation pattern and gene expression differences between iPSC lines from the same patient[75-78]. This could be due to a multitude of factors, including expression of reprogramming vectors, point mutations, and copy number variants generated in the reprogramming process[79]. Any induced genetic or epigenetic abnormalities are of concern in the study of gene-environment interactions in PD since they may mask the effect of a patient's individual genetic variation. In order to account for these effects, karyotyping should be performed at minimum, and whole genome and bisulphite sequencing should also be considered. Furthermore, it should be noted that *in vitro* studies utilizing iPSC-derived cells are limited in interpretation because they lack complex extracellular environments, neuronal architecture, and glial interactions. However, there have been efforts to address these concerns through development of mixed neuronal-glial cultures, but results have been inconsistent thus far[80]. Finally, since PD

is an age-related disease, newly differentiated neurons from iPSCs fail to simulate long-term simulate age-related phenotypes.

The promise of PD patient-derived iPSCs for personalized medicine and risk assessment

PD has the potential to benefit tremendously from the wide utilization of iPSC technology. This system permits the study of gene-environment interactions utilizing cellular subtypes derived from patients afflicted with PD. iPSC technology provides a critical link between epidemiological studies and animal, cellular, and computational models. This feature has potential for direct clinical application, as iPSC-derived neurons can be used to design customized neuroprotective strategies and recommendations for patients at the preclinical or early clinical stages of PD. In a parallel fashion, drug development can be accelerated by the development of high-throughput assays utilizing iPSC-derived neurons. Although PD-derived iPSCs share the limitations of other *in vitro* model systems, the fact that they are derived from patients with a clinical diagnosis offers the ability to explore processes such as oxidative stress, protein aggregation, and mitochondrial dysfunction in response to toxicants without a full understanding of the genetic factors underlying PD pathogenesis.

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CANDIDATE
REVIEWS

Circuit Refinement in the Developing Nervous System: Uncovering the Molecular Mechanisms that Destabilize the Synapse

Tyne Miller

Developing neural circuits undergo extensive refinement, characterized by the dynamic addition and removal of synapses. Localization of synaptic connections is critical for circuit architecture and information flow. Incorrect or excess synapses are eliminated to produce the precise cellular connections that are characteristic of mature nervous systems. This process has been observed across phylogeny, suggesting that the underlying mechanisms may be evolutionarily conserved. Studies of the vertebrate neuromuscular junction and visual circuitry have defined some overall themes in synapse refinement; the process is modulated by circuit activity and is commonly characterized by competition between inputs. The molecular networks that connect circuit activity to refinement are largely unknown, suggesting the need for a simpler model system. This review will examine the current understanding of synaptic refinement and introduce C. elegans as a model system to examine the molecular underpinnings of this complex, conserved process.

Keywords: *Synapse refinement, synaptic pruning, input elimination, synaptic remodeling, activity dependence, C. elegans, synapse disassembly, ubiquitination*

Critical period: Time interval during the development of an organism characterized by increased plasticity in the nervous system and an increased sensitivity to stimuli.

Heterochronic gene: Gene that controls the timing of development.

Introduction

During development, nervous systems create many more synapses than will be maintained at maturity[1]. It is unclear why this happens, but reduction involves large-scale removal of redundant synapses. The elimination of functional synapses is a hallmark of circuit refinement[2-4]. Studies of the neuromuscular junction (NMJ) have demonstrated that functional synapses are eliminated during circuit refinement[2-5]. Although it seems likely that functional synapses are also dismantled in the central nervous system, clear evidence of this phenomenon has been difficult to acquire[2-5]. Studies at the cerebellum show that incomplete elimination results in coordination defects in mice, suggesting that synapse elimination is important in creating functional neural circuits[6-8]. Synaptic refinement ranges from the disassembly of individual synapses to the complete removal of all connections between a presynaptic cell and its postsynaptic target[4,6]. The refinement of neural circuits has been observed in diverse organisms ranging from metamorphosis in insects to the development of the mammalian brain, indicating its evolutionary importance[1,9].

Synapse refinement is tightly regulated temporally[10-11]. Synapse elimination occurs in adults during injury or disease; however, this process is much more prevalent in the developing nervous system[12-13]. Extensive refinement occurs during critical periods of vertebrate development in the visual system, auditory system, cerebellum, and skeletal muscle[10-11]. Additionally, refinement is controlled temporally during the larval development of the invertebrate *C. elegans,* where the heterochronic gene *lin-14* controls the timing of GABAergic circuit remodeling[14].

Much of what we know about the process of synapse refinement has come from studies of the vertebrate NMJ, due to its simplicity and accessibility[15]. Models of central nervous system (CNS) refinement include the visual system circuitry and climbing fibers in the cerebellum[16]. Additionally, the simple nervous systems in *Drosophila* and *C. elegans* provide excellent models for molecular and genetic

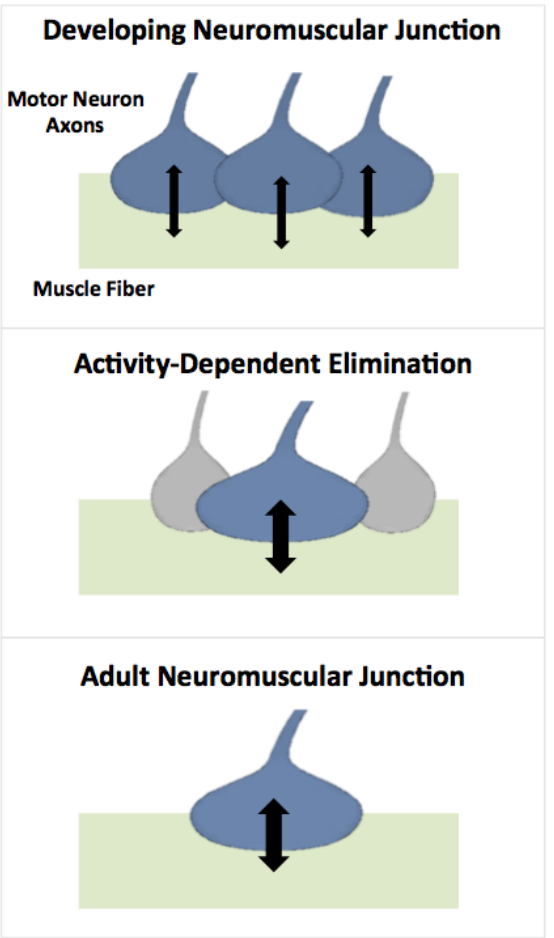

Figure 1: *Activity-dependent elimination at the neuromuscular junction (NMJ).* Initially, axons from multiple motor neurons innervate a single muscle fiber. Activity-dependent elimination results in the strengthening of one synapse (blue), while the other inputs become weaker and are eliminated (gray).

manipulation[17-18]. Our current understanding from studying these diverse model systems has led to overarching themes in synapse refinement, highlighted below.

Synaptic Activity and Circuit Refinement

Early studies of the visual system demonstrated that electrical activity plays a critical role in the refinement of neural circuits[19-22]. Axons from the left and right eye project to the lateral geniculate nucleus (LGN) of the thalamus[23]. The thalamus sends projections to the visual, or striate, cortex, relaying information from the eyes[23]. In the mature nervous system, inputs from each eye are segregated into distinct segments in the LGN and cortex[23]. The compartments in the visual cortex, termed ocular dominance columns, were identified by the striped patterns seen on cortical sections after injecting dye into one eye[19-23]. In a classical experimental paradigm, vision was occluded in one eye of a developing cat[19,22]. Even when monocular occlusion was restricted to a short window of time, a loss of ocular dominance columns was observed in the visual cortex[19-24]. Additionally, the presynaptic arbors of the axons from the occluded eye were much smaller, whereas arbors from the non-deprived eye expanded[25-26]. Later studies found that the ocular dominance columns form before birth but can be lost during postnatal critical periods if circuit activity is interrupted[27]. Extensive refinement also occurs at the LGN[2]. The cells of the LGN are initially innervated by multiple retinal axons, but following eye opening, these synapses are eliminated so that only 1-3 inputs remain[2,23].

Similarly, a critical role for circuit activity at the vertebrate NMJ has been well characterized[28-29]. The NMJ consists of the presynaptic motor neuron and the postsynaptic muscle fiber, ensheathed by a Schwann cell[30]. The presynaptic neuron releases acetylcholine (ACh), which binds to postsynaptic ACh receptors located on the membrane of the muscle fiber[30]. Initially, multiple axons from different motor neurons synapse with the same muscle fiber[23]. Excess inputs are eliminated over time, resulting in each muscle stabilizing innervation from only one motor neuron (**Figure 1**)[31-32]. During the elimination process, one input becomes stronger, whereas the other axons become weaker and retract[15]. Studies show that blocking activity results in defective elimination and increased numbers of inputs[15,28-29,31]. Additionally, higher levels of activity can induce input elimination in less time[15,31-33]. It appears that both presynaptic and postsynaptic activity play an important role in circuit refinement[15,31,35]. Studies of the rodent NMJ have demonstrated that modulating activity in the axon or muscle cell can influence synaptic elimination[15,34-35].

Data now suggest that activity may not be sufficient to promote input elimination; rather, it is proposed that the pattern of neuronal firing dictates synapse refinement. Mature motor neurons that innervate the same muscle fire asynchronously[36-37]. Interestingly, *in vitro* studies of the mammalian NMJ show that activity is synchronous early in development, prior to synapse refinement[36-37]. During development, synchronous activity is replaced by

asynchronous firing, and this occurs around the onset of input elimination[36-37]. The imposition of synchronous activity on the NMJ inhibits the input elimination[36-37]. This study suggests that differential activity is needed at the NMJ to stabilize the active synapses and to prune the less active inputs. Additionally, visual system refinement is sensitive to the pattern of activity. Studies show that flashing strobe lights into the eyes of goldfish and frogs during the critical period of visual circuitry refinement delays synapse elimination and thus fails to maintain ocular dominance columns[38-39]. These results correlate with the Hebbian paradigm in which neurons that fire together are strengthened and maintained, whereas noncoincident activity weakens connections[40-42].

Taken together, these studies demonstrate that patterned circuit activity is a conserved player in remodeling the nervous system. Interestingly, not all synaptic refinement events are modulated by activity. Hormonal signaling controls metamorphosis in insects, whereas axon guidance molecules can mediate retraction in the vertebrate CNS[17,43]. Additionally, refinement can occur at electrically silenced NMJ synapses *in vitro*[44]. These results are important because they show that both activity-dependent and independent pathways can modulate synaptic remodeling.

Competition at the Vertebrate Neuromuscular Junction

Studies at the vertebrate NMJ have been critical in demonstrating that multiple synaptic inputs compete with one another to innervate a target muscle fiber[25,31,45]. *In vitro* studies showed that in muscle fibers that are innervated by two motor neurons, stimulation of one neuron led to suppression of inputs from the other neuron[35]. The mechanism of this effect was explored *in vivo* by genetic ablation of choline acetyl-transferase (ChAT) in a subset of motor neurons, thus selectively depleting biosynthesis of the neurotransmitter acetylcholine[31]. When competing with wild-type neural inputs, the ChAT-depleted inputs lost the competition to innervate[31]. This study demonstrates that more active inputs out-compete weaker inputs to stabilize innervations with target muscle. One motor neuron may lose innervation at one site but out-compete and stabilize connections at other sites, suggesting a mechanism to bias connections for maintenance or removal[46]. Interestingly, the competition of motor neurons is reversible[45,47-48]. Increasing the activity in weaker inputs can cause initially "losing" motor neurons to "win" the competition[45]. Recent studies demonstrate that axons undergoing elimination will reverse their fate if the innervating axon is excised[45]. This finding suggests that the pruning process is not all-or-none but rather is a continually driven process[30]. Another interesting characteristic of elimination is that at no time in the refinement process are muscle fibers without innervation, indicating that cellular mechanisms may exist to detect synaptic density and ensure all muscle fibers maintain input from at least one motor neuron[49].

Studies of competition at the vertebrate NMJ have led to the idea that axons may be competing for a limited trophic factor released from the post-synaptic muscle[4,15,30]. The inputs with access to more trophic factor are stabilized, whereas inputs receiving less trophic factor are eliminated[15]. Studies show that trophic support is required for the maintenance of synapses[50]. The loss of the neurotrophin-4 ligand or its receptor TrkB promotes synaptic elimination at the muscle and cerebellum[51-52]. Overexpression of neurotrophin-4/5 and brain derived neurotrophic factor (BDNF), ligands of the TrkB receptor, prevent elimination and promote synapse stabilization in the visual circuitry of cats[53-54]. Additionally, studies at the NMJ suggest that the postsynaptic muscle must have a mechanism to communicate with the presynaptic motor neurons; however, this signal has not yet been identified[15]. It remains speculative that neurotrophins are the retrograde signal at the NMJ that stabilize a single motor neuron while destabilizing others. Taken together, these studies suggest that the local control of trophic factors may contribute to the stabilization or removal of competing axons.

It is important to note that not all synapse elimination events involve competition between multiple inputs. At the *Drosophila* NMJ, motor neurons innervate target muscle without competing against other inputs; however, the size of the synapses increases greatly over time[55]. In coordination with this synaptic growth, disassembly occurs in restricted areas and the postsynaptic muscle expands[30,55-56]. Additionally, competition between inputs does not appear to dictate synapse disassembly in the refinement of *C. elegans* GABAergic circuitry although this process is activity-dependent[9,57]. These results imply that competition between inputs may be characteristic of the more complex vertebrate nervous systems, while activity-dependent refinement is conserved in simpler invertebrate neural circuits.

Uncovering the Molecular Mechanisms of Synaptic Disassembly

It is unclear what molecular mechanisms connect activity in the nervous system to the cellular constituents that

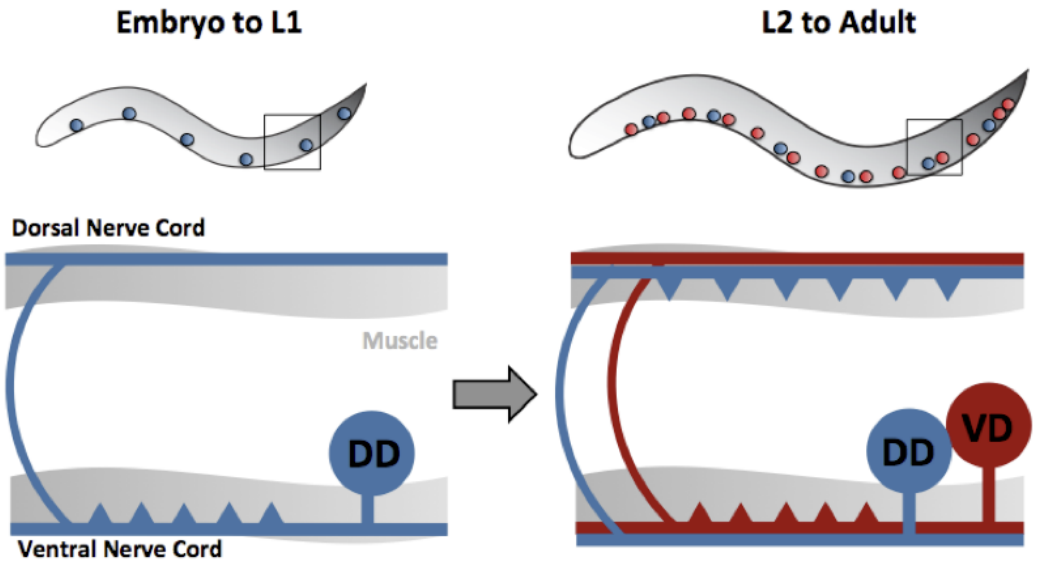

Figure 2: *Remodeling the GABAergic motor circuit in C. elegans.* Dorsal D (DD) motor neurons initially innervate ventral muscle (blue triangles), then relocate these synapses to dorsal muscles following the first larval stage (L1). The Ventral D (VD) motor neurons are generated in the second larval stage (L2) and synapse with ventral muscles (red triangles).

physically dismantle synapses. One hypothesis involves the activity-dependent destabilization of synapses by the ubiquitin proteasome system (UPS)[58-63]. The proteasome regulates protein concentrations in the cell and removes defective proteins by degradation[62]. Proteins are targeted for proteasomal destruction by ubiquitin molecules[62]. This process involves three enzymes: the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin ligase (E3)[62]. The E3 ubiquitin ligase is responsible for substrate specificity[62]. Studies show that ubiquitination of both pre- and post-synaptic proteins occur at the synapse[58-63]. Interestingly, the ubiquitination of proteins at the synapse also appears to be modulated by activity[59-60]. A study in isolated vertebrate hippocampal neurons shows that in response to activity, the UPS degrades PSD-95, a scaffolding protein in the postsynaptic cell[58]. Activity-induced ubiquitination of PSD-95 induces the internalization of AMPA receptors. Thus, this study connects circuit activity to protein turnover and molecular changes at the synapse[58]. Another study shows that Shank, GKAP and AKAP79/150 postsynaptic scaffolds selectively undergo activity-dependent ubiquitination[59]. The selective ubiquitination of scaffolding proteins and their associated proteins could be a mechanism to regulate synapse stability in response to activity[59]. Additionally, it was found that during synapse removal, distinct scaffolds of proteins are eliminated at different times, showing that differential regulation of groups of proteins play an important role in the process of disassembly[64].

Examining the activity-dependent ubiquitination of presynaptic proteins has led to similar findings[60-61]. Studies at the *Drosophila* NMJ show that proteasome inhibitors strengthen synaptic transmission through up-regulation of a vesicular priming component DUNC-13[60]. An additional study in *C. elegans* proposes an interesting model for protecting synapses from ubiquitination and destruction[61,65]. The immunoglobulin protein SYG-1 interacts with the E3 ubiquitin ligase SKR-1[61]. This interaction blocks the binding of SKR-1 to adaptor protein SEL-10, needed for ubiquitin-mediated target selection, thus blocking presynaptic ubiquitination in this area of the neuron[61]. This study also suggests that in different areas of the same axon that lack SYG-1, SKR-1 is free to join the active ubiquitin complex and dismantle synapses[61]. This model introduces a mechanism by which the presynaptic neuron can spatially dictate synaptic stabilization along a single axon[61]. Taken together, these studies demonstrate that ubiquitination of pre- and post-synaptic components may act as a mechanism connecting activity to the cellular processes that destabilize the synapse. More studies will be necessary to understand what specific proteins are targeted for degradation in these systems and how the loss of targeted proteins affects synaptic stability.

Circuit Remodeling in *C. elegans*: A Model of Synaptic Removal

While the examination of vertebrate systems has been helpful to our understanding of synaptic remodeling, it is becoming more apparent that refinement is complex and

COUP-TFII: (chicken ovalbumin upstream promoter transcription factor-2) highly conserved transcription factor involved in patterning the nervous system in addition to controlling development of multiple organs in the body.

may involve the coordination of multiple cellular processes. Therefore, there is a demand for a simplified model to examine how different conserved pathways are acting with one another to regulate such a complex event. The nematode *C. elegans* is widely used for its ease of genetic manipulations and its highly conserved genome. Interestingly, a subset of neurons in the worm undergoes expansive remodeling during development[9]. The GABAergic Dorsal D (DD) motor neurons are born embryonically and make synapses onto ventral muscles[9]. At a critical window of development, specifically between the first (L1) and second (L2) larval stages, DD synapses with ventral muscles are removed as new DD connections are established with dorsal muscles[9]. Coincidentally, Ventral D's (VDs) are born and innervate the ventral muscle (**Figure 2**)[9]. The UNC-55/COUP-TFII transcription factor functions in VDs to inhibit remodeling [66-67]. When UNC-55 is genetically ablated, the VDs ectopically remodel, and conversely, over-expression of UNC-55 in DDs blocks remodeling[66-68]. Therefore, the UNC-55 transcription factor is necessary and sufficient to inhibit the GABAergic motor neuron remodeling program. Activity in the form of neurotransmitter release also modulates this process. Mutants that decrease synaptic vesicle fusion show delayed DD remodeling, whereas mutants that increase neurotransmitter release demonstrate precocious or early remodeling[69]. Recent work demonstrates that this process is very complex. A microarray study was performed to uncover the targets of UNC-55, with the assumption that these would be candidate synaptic remodeling genes[18]. This approach identified 49 candidate genes with gene ontology categories ranging from ubiquitin regulation, calcium binding proteins, ion channels, enzymes, extracellular matrix components, transcription factors, cytoskeletal components, and proteins involved in neurotransmission[18]. This study demonstrates the complex nature of the remodeling process and yields candidate genes that may be conserved in refinement of the mammalian central nervous system.

Conclusions

The regulation of synaptic refinement is complex; however, with the use of model organisms we are developing deeper insight into the molecular mechanisms that govern this form of neural plasticity. Despite the complexity of the mammalian central nervous system, we have seen that synaptic refinement relies on the activity of neural circuitry and is characterized by competition between multiple inputs. Additionally, it appears that mechanisms to destabilize the synapse may play a role in activity-dependent synapse disassembly. It is unclear how the nervous system is able to coordinate the assembly, disassembly, and reassembly of a vast number of synapses during development. This suggests that the nervous system requires mechanisms to control synapse formation and retraction, in addition to mechanisms that balance the two. Much work will be necessary to elucidate the molecular constituents that refine functional neural circuitry. The utilization of model organisms with simplified nervous systems and malleable genetics will be of great value in exploring these intriguing questions.

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Roles of Serotonin Signaling in Embryonic and Postnatal Neurogenesis

Elaine Ritter

Serotonin (5-hydroxytryptamine, 5-HT) is perhaps the most well-studied of the classical neurotransmitters and has been implicated in many behavioral adaptations as well as essential physiological functions. In addition to its role in these processes, 5-HT is also a vital regulator of development and begins acting as early as the end of gastrulation and continues throughout postnatal development. A rich body of research has established that 5-HT is intimately involved in the survival, proliferation, migration, differentiation, and maturation of neurons, and the formation of functional synapses. This review surveys experiments that have demonstrated the role of 5-HT signaling in each of these processes, most of which are mediated by G-protein coupled receptor signaling cascades. However, 5-HT3, the only ligand-gated cation channel member of the 5-HT receptor gene family, has been understudied in the context of development. Evidence presented here suggests a significant role for the 5-HT3 receptor in neurogenesis, but much is still left to be learned about the mechanisms by which it acts. The information gained from the study of 5-HT signaling in neurogenesis has important implications for a more thorough understanding of the development of the central and peripheral nervous systems.

Keywords *Serotonin, serotonin receptors, neurogenesis, development, signaling cascade, molecular biology*

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is an evolutionarily ancient molecule[1] that is critical for the modulation of many physiological and behavioral adaptations. Among these adaptations are appetite, gastrointestinal function, nociception, sexual behavior, mood, learning and memory. The role of 5-HT in these processes has been extensively studied for decades. Outside the realm of classical neurotransmission, 5-HT is considered to be a key regulator of neurogenesis through neurotrophic effects[2,3], and this notion is well supported in the literature. 5-HT expression is highly abundant in the developing central and peripheral nervous systems[4]. Additionally, serotonergic neurons housed in the brainstem are among the earliest born[5,6] and they innervate most regions of the brain[7]. One of the foundational experiments identifying 5-HT in neurodevelopment demonstrated that pharmacological depletion of 5-HT during rat embryogenesis results in impaired neural differentiation in the brain[8]. Since then, advances in molecular biology and pharmacology have permitted study of the modulation of neurogenesis by 5-HT during embryonic and postnatal development, specifically by the activity of 5-HT receptors and the 5-HT transporter, SERT. This review highlights research that attests to the importance of 5-HT signaling throughout neurogenesis in mammalian development and identifies questions in the field that remain unanswered.

Neurogenesis:
The process by which neurons are generated from neural stem and/or precursor cells.

Differentiation:
The developmental process of neural progenitors acquiring the genetic and phenotypic characteristics of their terminal neuronal fates.

Serotonin in the Modulation of Survival, Proliferation and Differentiation

Numerous studies support the hypothesis that 5-HT promotes the proliferation and survival of neuronal progenitors early in embryogenesis. One way 5-HT acts in these processes is by mediating the activity of glycogen synthase kinase-3β (GSK3β). GSK3β

Wnt signaling pathway: Highly conserved cellular signal transduction pathway that plays a crucial role in embryonic and fetal development; known downstream effects include modulation of gene expression, cytoskeletal dynamics, and intracellular calcium levels.

Specification: Also referred to as lineage segregation or divergence, the means by which neural precursors are assigned neuronal identities but have yet to become determined or undergo differentiation.

acts as a negative regulator of neurogenesis by inhibiting β-catenin in the canonical Wnt signaling pathway. Inhibition of GSK3β allows β-catenin to enter the nucleus and activate target genes that promote the survival and proliferation of neuronal progenitors[9]. The effect of 5-HT on GSK3β activity was first observed in the mouse brain—upon administration of d-fenfluramine, a SERT inhibitor, GSK3β becomes phosphorylated and subsequently inhibited in the cerebral cortex and hippocampus[10]. A later experiment showed a similar effect in animals lacking functional tryptophan hydroxylase-2 (Tph2), a key enzyme in neuronal 5-HT synthesis: Tph2 null mice also display phosphorylated, inhibited GSK3β[11]. In both experiments, inhibited GSK3β results in perpetuation of the neural stem cell profile, such that survival and proliferation programs are maintained. Interestingly, the selective serotonin reuptake inhibitor (SSRI) fluoxetine has been proposed to mediate its antidepressant effects by fostering neurogenesis in the hippocampus[12-14]. This hypothesis is supported by the observation that fluoxetine increases phosphorylation and inhibition of GSK3β, thereby promoting survival and proliferation[15,16]. Further experimentation revealed that inhibition of GSK3β occurs by signaling through the serotonin type 1A receptor (5-HT1A). Specifically, 5-HT1A signaling stimulates the kinase activity of Akt[17,18], which is known to phosphorylate and inhibit GSK3β[19-21]. In favor of this mechanism, it was shown that 5-HT1A-specific antagonists block phosphorylation of GSK3β by Akt[10]. Collectively these findings demonstrate that 5-HT1A signaling causes inhibition of GSK3β, stimulating survival and proliferation in neurogenesis.

While there is no evidence substantiating a direct role for 5-HT in specification of neurons, there is support for 5-HT regulating mechanisms of differentiation. Contradictory to its influence on GSK3β inhibition, 5-HT1A signaling mediates effects that oppose the proliferation and survival of neuronal precursors by promoting neuronal differentiation. Autoinhibition of the 5-HT1A receptor moderates serotonergic neuronal differentiation in the developing raphe nucleus, as evidenced by increased serotonergic density in this region in animals lacking 5-HT1A[22]. It has also been noted that the 5-HT1A receptor is expressed early in the mouse fetal brain, and over developmental time its autoinhibitory activity results in decreased 5-HT1A expression as serotonergic neuronal differentiation increases[23]. Corroborating these results, other experiments showed that the absence of SERT leads to excessive stimulation of 5-HT1A receptors and subsequent inhibition of serotonergic neuronal development[24]. Aside from serotonergic neurons, 5-HT signaling also stimulates differentiation of glutamatergic neurons in the embryonic cerebral cortex[25], demonstrating that 5-HT affects the differentiation of multiple neuronal subtypes. In an effort to tease apart the mechanisms underlying these effects, work in cultured cells from the developing brainstem and cortex of fetal mice showed that 5-HT1A activity stimulates the release of S100β from astroglial cells expressing this receptor[26-29]. S100β, when secreted from astroglial cells and taken in by neighboring neuronal progenitors, is anti-apoptotic[30,31] and promotes neuronal differentiation[32]. This factor appears to be involved in regulating the stability of tubulin in the construction of microtubules, thus serving to help stabilize the neuronal cytoskeletal architecture required for differentiation[33]. These collective reports indicate that the 5-HT1A receptor plays opposing roles in neurogenic processes and further study is needed to dissect its mechanisms of action in various settings.

The influence of 5-HT signaling on survival, proliferation and differentiation of neuronal progenitors is not unique to the central nervous system. While 5-HT2B promotes survival in cortical neuronal progenitors[34,35], it also modulates survival and differentiation during peripheral neurogenesis. Depletion of 5-HT2B at the onset of neurulation in mice results in precocious neuronal differentiation

and failure of cranial neural crest cells to migrate to the periphery[36]. In the enteric nervous system (ENS), it was shown by Michael Gershon's group that this receptor is important for initiating differentiation of enteric neurons[37]. Moreover, they have also published studies demonstrating the requirement of 5-HT4 for the generation and survival of neurons in the developing postnatal enteric nervous system[38]. By ablation of Tph2 gene expression, they showed that 5-HT is necessary for the proper differentiation and maintenance of dopaminergic neurons in the gut[39]. These experiments collectively emphasize the significance of 5-HT signaling in the development of several neuronal populations, although the underlying molecular mechanisms have yet to be dissected. How 5-HT functions in the neurodevelopment of other visceral organs outside the ENS is still largely unknown. Clearly, the means by which serotonin signaling impacts the beginning stages of neurogenesis are highly diverse and the downstream effects of several 5-HT receptors vary throughout the course of development.

Serotonin in Neuronal Maturation and Synaptogenesis

An extensive body of literature reports the effects of 5-HT signaling on the maturation of the neuronal phenotype following differentiation and the formation of functional synapses. The involvement of 5-HT in neurite outgrowth and dendritic arborization is especially well-supported by numerous studies. In embryonic development of the mouse raphe nucleus, loss of the 5-HT1A receptor results in an increase in neurite number and length[22]. A comparable autoinhibitory effect of this receptor was noted in cultured fetal rat cortical neurons[40]. However, deprivation of 5-HT during postnatal rat development ultimately leads to deficient dendritic branching on granule cells of the dentate gyrus, which can be rescued with a 5-HT1A receptor agonist[41]. As described earlier in the context of proliferation and differentiation, it appears that 5-HT1A activity has varying effects either by promoting or inhibiting neuritic outgrowth and maturation. Other 5-HT receptors are also influential in neuronal maturation. For example, in fetal mice, enhanced neurite outgrowth is seen when 5-HT1B receptors expressed in thalamic neurons are stimulated[42]. Similarly, detailed studies have revealed that the 5-HT7 receptor activity regulates neuronal architecture in the construction of cortical columns as serotonergic inputs connect to Cajal-Retzius cells[43]. Coupling between stimulated 5-HT7 and the Gα-12 protein activates the RhoA and Cdc42 signaling cascades. When activated by Gα-12, these factors promote and impede, respectively, neurite outgrowth and growth cone motility[20,44-46]. Conversely, 5-HT4 activity, by coupling to the heterotrimeric G13 protein and activating the RhoA signaling cascade, inhibits neurite outgrowth and cell-rounding during neurogenesis in the hippocampus[47,48]. Analogous to the complexity of actions seen in survival and proliferation, the opposing effects of 5-HT in neurite outgrowth and maturation attest to the diversity of the roles this neurotransmitter can play in multiple neurogenic processes.

In addition to its influence on dendritic morphology, 5-HT has also been implicated in moderating axon guidance mechanisms in development. Work from Levitt and colleagues in recent years have clearly demonstrated a unique role for 5-HT signaling in guidance of thalamocortical axons in the developing forebrain of rodents and humans. Using 5-HT1A/1D receptor-specific drugs, they found that activation of the $G_{i/o}$-protein signaling pathway through these receptors, which inhibits adenylyl cyclase and decreases intracellular levels of cAMP[49], causes the axonal attractant netrin-1 to become a repulsive cue for migrating axons[50]. Additionally, segregation of axons in developing thalamic sensory projections relies on appropriate levels of 5-HT signaling in the early postnatal brain—overstimulation of 5-HT1B receptors results in cytoarchitectural aberrations of somatosensory projections to the thalamus and thalamic projections to the barrel field cortex[51]. All the studies reported here emphasize the importance of 5-HT signaling in neuronal maturation processes.

The role of 5-HT signaling in neurogenesis extends from neuronal maturation and axon guidance to the formation of synapses during embryonic and postnatal development. Recalling the early neurogenic effects of 5-HT signaling on s100β expression, 5-HT continues to act through s100β to support synaptogenesis and synaptic plasticity. Transient depletion of 5-HT during rat postnatal brain development leads to a subsequent loss of s100β, which ultimately results in a thinning of synaptic density in the hippocampus of adult rats[52]. The consequence of 5-HT and s100β loss during this time is permanent, such that even after restoration of endogenous 5-HT these synapses cannot be reformed in adulthood. The actions of 5-HT signaling on s100β not only affect synapse formation but also plasticity. When s100β is lost early in mouse development, the remaining hippocampal synapses are less likely to adapt to learning and memory formation compared to control animals[53]. It

is important to recognize that the effects of 5-HT signaling during developmental neurogenesis continue to impact adult brain structure and function.

A Potential Role for the 5-HT3 Receptor in Neurogenesis

Throughout this review of serotonin's modulation of neurogenesis, the focus has been on the members of the 5-HT receptor family that are G-protein coupled receptors. The 5-HT3 receptor is the only serotonin receptor that is a ligand-gated ion channel[54,55], and there are several lines of evidence that implicate an important role for 5-HT3 in central nervous system development. Loss of 5-HT3 activity in cortical development results in decreased levels of reelin. The expression of reelin, an extracellular matrix glycoprotein involved in neurogenesis, has been demonstrated in Cajal-Retzius cells in layer I of the cortex in mice[56]. Reelin expression in this cell population is dependent upon the reception of excitatory input through 5-HT3A receptors[57]. It has also been demonstrated that reelin helps coordinate the migration of sympathetic preganglionic neuronal progenitors to the spinal cord in fetal development[58], which suggests a role for 5-HT signaling in this system. 5-HT3 receptors have also been shown to be involved in the migratory patterns and maturation of GABAergic interneurons in mouse cortical development. Application of a 5-HT3 agonist causes this cell population to have abnormally long neuritic processes with few branches, while a 5-HT3 antagonist results in the formation of numerous, short processes and failure to migrate to the cortical plate[59]. Moreover, fluoxetine has been demonstrated to functionally suppress the activity of 5-HT3 receptors[60], and very recently it was discovered that prenatal exposure to fluoxetine reduces dendritic complexity by nearly 50% in pyramidal neurons of the cortex in mice[61]. This receptor's actions in neurogenesis extend into the construction of neuronal architecture. Elegant experiments have demonstrated an interaction between the intracellular portion of the 5-HT3 receptor and F-actin, implying that 5-HT3 modulates cytoskeletal structure during neuronal migration and maturation[62]. 5-HT3 activity appears to mediate multiple neurogenic processes throughout the course of neurodevelopment—yet, the causal mechanisms underlying the function of 5-HT3 are poorly understood.

It is widely known that regulation of calcium flux in neural progenitors is required for proper nervous system development to occur. Interestingly, presynaptically localized 5-HT3 is permeable to calcium in neurons of the corpus striatum, hippocampus and amygdala[63,64]. 5-HT3 receptors have enhanced calcium permeability in several neuroblastoma cell lines as well[65,66]. Given this information, it is plausible that 5-HT3 exerts its effects on neurogenesis via calcium signaling. Research supporting this hypothesis has only been conducted fairly recently. PC12 cells express 5-HT3, and an increase in intracellular calcium levels was observed in response to treatment with a 5-HT3 agonist[67]. This effect led to Nerve Growth Factor (NGF) upregulation, resulting in neurite outgrowth and differentiation, and is blocked by a 5-HT3 antagonist. Surprisingly, the L-type calcium channel antagonist nifedipine also inhibits the 5-HT-induced increase in intracellular calcium and its stimulation of NGF. The response to nifedipine suggests that 5-HT3 stimulation likely affects the activity of voltage-gated calcium channels, providing another mechanism by which 5-HT3 receptors are responsible for regulating calcium flux in developing neurons. This hypothesis is especially intriguing, as the calcium-dependent promotion of Brain Derived Neurotrophic Factor (BDNF) transcription by the CREB pathway is mediated by L-type calcium channels[68-70]. It was also shown that 5-HT3 receptors are expressed transiently in the glutamatergic granule cells of the developing cerebellum, where they were shown to be critical for promoting plasticity during synaptogenesis of Purkinje cells and parallel fibers[71]. The authors of this work postulate that 5-HT3 may be mediating its effects via control of calcium flux, either through 5-HT3 itself or its activation of L-type calcium channels, but this possibility has not yet been pursued.

In contrast to the intriguing studies conducted in the CNS, the role of 5-HT3 in PNS development is almost entirely unknown. In 1996, Johnson and Heinemann reported expression of 5-HT3 in the neural crest cells of rat embryos aged 15 days post coitus (dpc), including sympathetic and parasympathetic ganglia of the enteric nervous system and the dorsal root ganglia (DRG)[72]. This observation is corroborated by demonstration of 5-HT3 gene expression in the DRG of 14.5 dpc fetal mice[73]. There has been no follow up on these findings, so the function of 5-HT3 throughout neural crest development remains unclear. As mentioned previously, the development of some aspects of the PNS is poorly studied; among these is the innervation of the lower urinary tract (LUT). Proper 5-HT3 function is critical for maintaining the autonomic innervation of the LUT in adult mice[74], but its role in the development of this system has not yet been explored. Based on the importance

of 5-HT signaling in neurogenesis and the preliminary observations outlined here, there is strong support for the hypothesis that signaling through the 5-HT3 receptor influences neural crest development and the innervation of the LUT.

To investigate the function of 5-HT3 in neural crest cells, a wide variety of tools is available. Transgenic reporter mouse lines[75] allow visualization of 5-HT3 expression throughout embryonic and postnatal development. A 5-HT3 knockout line exists[76] and would be valuable to study the effects of loss of this receptor on neural crest survival, differentiation and migration to the LUT. *In vitro* study of mechanisms by which 5-HT3 mediates neuronal specification of neural crest progenitors is possible with numerous drugs affecting 5-HT3 activity[4]. Pharmacological agents may also be used to tease apart the downstream signaling cascades regulating neurogenesis that are stimulated by 5-HT3. Additionally, emerging technology facilitating imaging of calcium flux in live cells permits examination of the functionality of 5-HT3 in neurogenesis[77]. These and other molecular biology techniques will allow researchers to begin to elucidate the role of 5-HT3 signaling in PNS neurogenesis.

Conclusions

A broad body of evidence, including studies described here and others not mentioned, underscores the importance of 5-HT signaling in multiple processes that comprise neurogenesis. 5-HT has been shown to be intimately involved in the survival, proliferation and differentiation of neuronal progenitors. Additionally, 5-HT functions to modulate the migration of differentiating neurons, the augmentation of neurites, and the construction of synapses and cellular architecture. The diversity of the processes regulated by 5-HT is reflected in the myriad signaling mechanisms by which 5-HT acts via its receptors. In fact, the same receptor can even serve oppositional functions throughout the course of development, as is the case with 5-HT1A and 5-HT7. While some of the signaling cascades mediating these processes have been dissected, much work remains to be done in order to discover the ways in which 5-HT signaling is able to take on so many roles throughout the course of neurogenesis. Especially compelling for future study is the 5-HT3 receptor, the only ligand-gated ion channel in the serotonin receptor family. Several publications implicate a significant role of 5-HT3 in neurogenesis—however, no one has yet assembled the pieces of the puzzle to understand precisely the ways by which this receptor affects neurogenic processes. The subject of 5-HT3 in peripheral neurogenesis is still largely untouched. Fortunately, pharmacological and molecular tools currently available make it possible to investigate how 5-HT3 guides neurogenesis in embryonic and postnatal development.

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The Role of the Principal Sensory Nucleus in Discriminative Touch

Eva Sawyer

Specialized facial somatosensory organs have evolved in diverse groups of animals, and the sense of touch that these organs transduce is important for normal behavior. The principal sensory nucleus of the spinal trigeminal complex is the first relay for facial discriminative touch in the central nervous system. Much of the work done on this nucleus is done in rodents, where the ability to trace the central representations of whiskers follicle innervation has been a useful tool for experimenters. Questions remain about the role of the nucleus, from uncertainties about the basic anatomy to its role in forming the disproportionate representation of the body seen in the cortical somatosensory maps. Comparative neurobiology points out that some non-rodent animals with specialized trigeminal somatosensory organs, such as the star-nosed mole, have a much larger principal sensory nucleus than one would expect for a mammal of their size. Complementing rodent work with studies on these species has the potential to help resolve puzzles about the entire spinal trigeminal complex, and the principal sensory nucleus in particular.

Keywords: *Somatosensory, trigeminal, segmentation, principal sensory nucleus, Barrels, star-nosed mole*

Introduction

Facial somatosensory specializations help animals navigate their world. Examples include whiskers on the face rodents and seals[1, 2], corpuscles of Herdst and Grandry on the beak of ground-probing birds[3, 4], push-rod receptors on the bills of montremes[5], integumentary sense organs on the jaws of crocodilians[6], and Eimer's organs on the noses of talpid moles[7]. These adaptations are associated with exploration, foraging and feeding[8, 9]. The co-evolution of sense organs and central processing centers is a theme in neurobiology[10, 11]. Accordingly, when researchers have looked at the first relay of the trigeminal somatosensory stream, these specialized trigeminal touch organs tend to be paired with central specializations[12-15].

The spinal trigeminal complex (STC) is the main target for the primary somatosensory receptors innervating the scalp, face and oral structures. The complex consists of the principal sensory nucleus (PrV) at the most rostral position and the spinal trigeminal nucleus (STN) more caudally. The latter consists of three subnuclei with subnucleus pars oralis (SpVo), subnucleus pars interpolaris (SpVi), and subnucleus pars caudalis (SpVc) found at progressively more caudal positions, respectively[16, 17]. SpVc merges with the dorsal horn of the spinal cord at its most caudal extent. The complex receives most of its sensory input from the somatosensory components of the trigeminal nerve (but also from the somatosensory components of the facial, glossopharyngeal and vagus nerves)[16]. Upon entering the brainstem, the trigeminal branch splits into an ascending branch that projects to the PrV and a descending branch that projects to the subdivisions of the STN.

Traditionally, there is a perception that in the somatosensory brainstem there is a division of labor so that the PrV mediates light touch sensation and the STN mediates pain[18]. In this view, the PrV is analogous to the dorsal column nuclei and the STN to the dorsal horn[19, 20]. Broadly, there is truth in the vital importance of the PrV to discriminative touch and the STN, especially the more caudal regions, to pain, but a strict view of non-converging labeled lines has weak support. This article will focus on the PrV, but it would be misleading to present the nucleus as if it were completely independent from the STN. Therefore, the STN will be mentioned where appropriate.

Form and Function

Ramón y Cajal illustrated trigeminal afferent axons branching to form an ascending path to the PrV and a descending path to the STN[21]. He writes that he cannot be sure that all of these axons bifurcate because he cannot rely on the

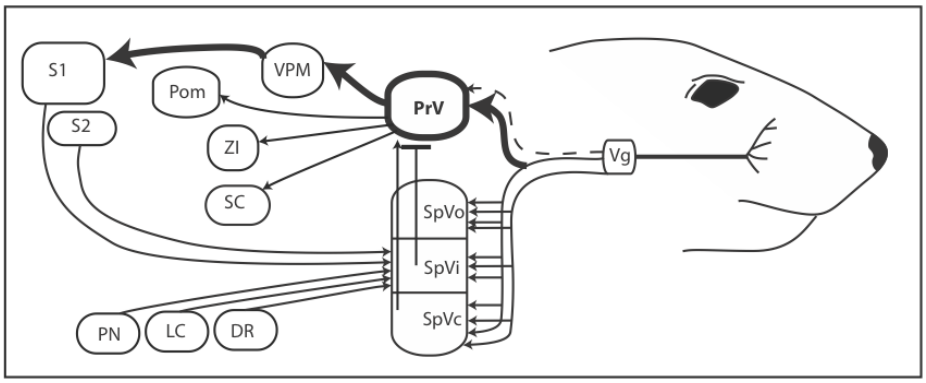

Figure 1: *Schematic of main connections of the principal sensory nucleus (PrV) and the regulatory input directed through spinal trigeminal nucleus pars interpolaris (SpVi).* The dotted line represents an unconfirmed class of sensory neurons projecting solely to the PrV. Thick lines represent the main pathway for low-threshold mechanoreception to the cortex. DR, dorsal raphe nucleus; LC, locus coeruleus; PN, Pontine nucleus; Pom, medial posterior nucleus; SC, superior colliculus; SpVc, spinal trigeminal nucleus pars caudalis; SpVo, spinal trigeminal nucleus pars oralis; ZI, zona incerta.

silver stain as an unbiased technique, but many, if not all, of the fibers he saw bifurcated. In a reinvestigation with silver stains Anstrom states he found the bifurcating fibers Ramón y Cajal reported, as well as descending non-bifurcating fibers projecting to only the STN[22]. He wrote that he did not observe, but could not rule out the possibility of, ascending non-bifurcating fibers projecting only to the PrV. Presumably, if these fibers exist, they would be mechanosensory axons that project exclusively to the PrV.

Another anatomist found such fibers. Windle, like Ramón y Cajal and Anstrom, used silver stains, but his studies were on fetal pigs instead of fetal and young mice[23]. He found three sub-populations of trigeminal afferents: 52% of axons bifurcated, 42% (mostly thin fibers) descended without bifurcating, and 6% (mostly thick fibers) ascended without bifurcating. He struggled to explain why other investigators did not observe large diameter non-bifurcating ascending fibers. One possibility he does not mention is that mice, with vibrissa used for whisking, and pigs, with a large glabrous nose used for rooting in soil, differ in their facial somatosensory specializations. Since the mechanoreceptors in these specializations differ, the animals may have different proportions of bifurcating and non-bifurcating trigeminal afferents.

Subsequent literature on ascending non-bifurcating axons is sparse, though the perception that this pathway exists is maintained in modern reviews and texts[24-27]. One method for studying projection patterns of sensory neurons in the brainstem is interaxonal injections of tracer combined with reconstruction of the labeled axons. This has been used in the STC with interaxonal injections of the neuronal tracers horseradish peroxidase or neurobiotin into the spinal trigeminal tract. Unfortunately, the injections are almost always at the level of SpVo or SpVi[28-33], a method which could not isolate an ascending non-bifurcating population. A less biased technique would use interaxonal injections upstream of the bifurcation, as done by Shigenaga et al.[34], or to inject a far-reaching tracer into single ganglion cells, as Jaquin et al.[35] piloted in a methods paper. Neither study found ascending non-bifurcating axons. The absence of this class could be because such neurons are not present in rodents. However, given that neurons with the ascending non-bifurcating branching pattern made up only 6% of trigeminal afferents in pigs, a combined sample size of 12 axons in rodents is unlikely to represent sufficient sampling to warrant a strong conclusion that these cells are absent in rodents.

Within the PrV, SpVo and SpVi the somatotopic map of the face is inverted so that afferents from the mandibular branch project dorsally, the maxillary branch intermediately and the ophthalmic branch ventrally[28, 31]. The anterior receptors are represented medially and the more posterior receptors,laterally. The arrangement in the SpVc is less well understood. Some studies find that the dorsal-ventral representation is unchanged but the medial-lateral representation is flipped, so that the anterior receptors are represented laterally and the more posterior receptors, medially[17, 36-38]. They also find that in SpVc there is rostral-caudal skew that results in the more rostral afferents being represented more rostrally in the nucleus. This arrangement is reminiscent of the rostral-caudal mapping of dermatomes found in the dorsal horn for the rest of the body. Despite this work, reviews sometimes depict the SpVc as organized like the other subdivisions[26], and recently the somatotopy of SpVc has reemerged as an issue[37]. It is noteworthy that there is still confusion about basic anatomy of the STC.

In addition to the main sensory input from primary sensory neurons, the PrV receives modulatory input from the STN and the cortex. Inhibitory GABAergic interneurons from the SpVi and excitatory glutamatergic interneurons from the SpVc project to the PrV[40]. These connections let the STN influence the sensitivity of the PrV[41]. Projections

from the primary and secondary somatosensory areas (S1 and S2) to the STC could also facilitate top-down reduction of PrV sensitivity[42-43]. In rodents, the cortex-STN-PrV pathway is thought to be particularly important during active whisking, when the somatosensory signals induced by body movement, and not by the characteristics of a substrate, are irrelevant. Such a circuit could explain how the sensitivity of the PrV is reduced during active whisking[44]. Other inputs are from the pontine tegmental nucleus[45-46], the raphe nucleus[47] and the locus coeruleus[48]. These likely reflect modulation of sensitivity based on the animal's level of alertness.

From the PrV, neurons project mainly to the contralateral ventral posterior medial nucleus (VPM) of the thalamus, which sends strong projections to S1[12,49-51]. This trigeminal lemniscal pathway is particularly notable in rodents because every station on this pathway (the PrV, the VPM and S1) has a pattern of cell-dense patches that correspond in a one-to-one manner with the whiskers on the animal's snout, termed barrelettes[17], barreloids[51], and barrels[12] in each location, respectively. We will return to barrels later in the essay. Other important direct projections are to zona incerta[52], the posterior medial nucleus of the thalamus[49] and the superior colliculus[53]. These projections likely contribute to the regulation of movement. The main connections of the PrV are summarized in **Figure 1**.

Electrophysiological work in a variety of species complements the above anatomical findings. As would be expected from the termination patterns of large diameter bifurcating axons branching to every STC subdivision, the PrV and the STN contain mechanosensory neurons[14,54-58]. Likewise, the anatomy shows that many small diameter fibers are non-bifurcating descending axons. If these are nociceptive c-fibers, then electrophysiological studies should find the STN enriched with nociceptive neurons. Indeed, electrophysiological studies that test for it fail to find nociceptive neurons in the PRV, but isolate them in STN[54,56,59-61].

Another promising area for animal studies is to use the power of genetic manipulations in model species to dissect the pathways of mechanoreceptors. For example, Li et al.[62] drove expression of reporter proteins in different classes of low-threshold mechanoreceptors in order to follow sensory neurons from the receptors in the skin to their projections in the dorsal horn. The same techniques are yet to be applied to neurons projecting to the dorsal column nuclei or to the STC. Both studies would be valuable. In the whisker pathway, it would be interesting to see if the different classes of low-threshold mechanoreceptors have unique projection patterns to the subdivisions of the STC, which has so far been undetected with electrophysiology and tract tracing.

Human case studies provide strong results that support the view of parallel pain and touch pathways. Lesions in the PrV cause deficits in touch sensation with sparing of temperature sense and nociception in the face[63], while lesions in the SpVc spare touch sensation but usually lead to the loss of nociception and temperature perception[18]. Thus, surgical damage to this area is a treatment for intractable orofacial pathogenic pain. With case studies such as these providing most of the background for the understanding of the human trigeminal system, a recent fMRI study was surprising. The study looked for changes in the blood oxygen level in humans who were experiencing noxious and non-noxious cutaneous and muscle stimulation to their face. As expected, noxious cutaneous and noxious muscle stimulation elicited changes in regions of the STN, but unexpectedly, the noxious muscle stimulation also elicited activation in PrV[64].

These unexpected results showing integration of pain and sensory information in the brainstem fit with a history of confusion about trying to connect the anatomical data, the electrophysiological data and now the fMRI data — which all show some integration of low-threshold mechanoreception and nociception — with human case studies, which show a strong division of touch and pain sensation between the nuclei[18,22,64]. One problem is that lesion studies rely on eliminating an entire node of a network, which provides strong but crude results. Another problem is that there is a push to make labeled lines for pain and touch explain the anatomical divisions of the STC, despite evidence that the divisions will not fit well into those categories. That debate, however, is beyond the scope of this article (see [65] for review).

Comparative neurobiology

Rodents have been seen as well suited for studies of the trigeminal touch pathway because their barrel system is more amenable to experimentation than non-patterned areas. The cortical barrels are impressive. For example, in rats, barrels cover 20% of S1, a total area of 9 mm^2 [66]. The development of this pattern is dependent on an intact PrV[67]. As othes have pointed out, it is odd that all the input for such a large cortical representation is funneled through a small PrV[54], 0.56mm^3 in the case of a rat[13]. Part of the resolution is that the cortex also receives connections from the

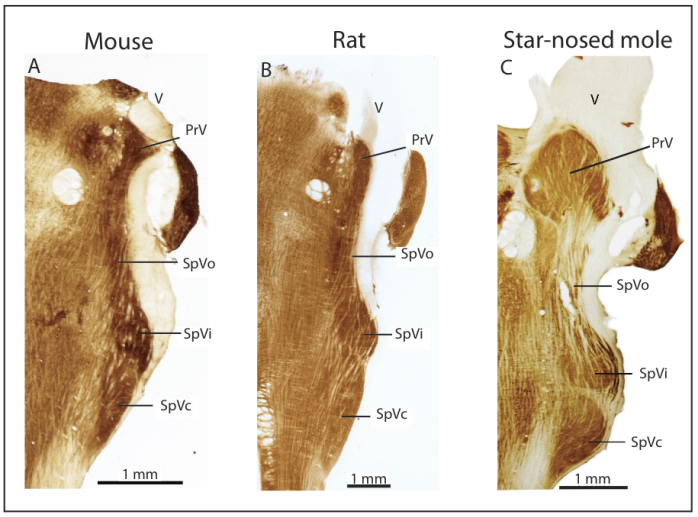

Figure 2: *Cytochrome oxidase stained sections of a mouse, rat and star-nosed mole brainstem cut in the horizontal plane.* Chosen sections maximized the volume of the principal sensory nucleus (PrV). Compared to the mouse and the rat, the star-nosed mole has a large PrV. SpVc, spinal trigeminal pars caudalis; SpVi, spinal trigeminal pars interpolaris; SpVo, spinal trigeminal pars oralis.

STN. Specifically, the caudal portions of SpVi project to the ventrolateral portion of the VPM in the thalamus. The VPM projects to the inter-barrel space, termed septa, in the cortex[68].

In rats, the volume of the SpVi is 1.66mm^3, almost three times larger than the PrV, and has more distinct barrelettes than the PrV[17,69]. Earlier we saw that the SpVi has a role in modulating sensory sensitivity based on directed movements of the whiskers. A large part of rodent exploratory behavior is active whisking — coordinated movements of the six muscles innervating each whisker pad to move the whiskers against the surface being examined[2,70-72]. All this suggests that rats and other rodents are good models for studying the SpVi, particularly because the subnucleus relates to active sensory behavior. But other animals may be better suited for investigating the PrV.

Comparative studies point out that in some species with elaborate somatosensory trigeminal sensory organs, the PrV is hypertrophied[9,14]. The case of the star-nosed mole is particularly informative because of the amount already known about its nervous system. The star consists of 22 fleshy appendages covered with Eimer's organs. Eimer's organs are composed of regular geometric arrangements of Merkel cell-neurite complexes, laminated corpuscles and free nerve-endings[7]. The star can be moved forward as a whole, and groups of appendages can be extended to bring the organ surface into contact with a substrate[73]. When moles forage, they rapidly move the star, touching it to the surfaces of the damp soil of their habitat to locate small food items in the mud[74]. There is a behavioral preference to use the two medial ventral rays when inspecting potential food items[75]. The neuroanatomical correlate of that preference is a larger representation of that ray in S1, and smaller receptive field size in the representation of the medial ventral rays than in the other rays[73,76]. The behavioral preference and the increased resolution suggest those rays as a somatosensory analog of the retina's fovea[75].

To put the size of the star-nosed mole PrV into perspective, the absolute volume of the PrV of a 55g star-nosed mole is larger than the PrV of a 274g rat — it is about 630% the size expected based on the proportions of a rat[13-14]. In comparison, the SpVi subdivision of the star-nosed mole is only about 50% larger than expected[9] (unpublished result). Acknowledging that the comparisons are crude and cover a wide taxonomic range, the results still show that the star-nosed mole has a large PrV even when compared to other somatosensory specialists (**Figure 2**).

The size of the PrV in the star-nosed mole is likely related to the exceptional spatial resolution of the star. Multi-unit receptive fields in the cortex average 0.82 mm^2 in the non-foveal part of the star and 0.52 mm^2 in the foveal regions, which are both smaller than receptive fields reported for primate fingertips[76-78]. If the PrV is the nucleus for fine touch, the extraordinary resolution of the star would be expected to distinguish this nucleus. There is already evidence that this is the case: within the PrV, as in the cortex, the medial ventral rays have a larger representation than the other rays. Interestingly, the greater size of the representation of the foveal rays in the cortex and PrV is not explained by greater innervation of these rays[14]. Combined with the smaller receptive fields in foveal than in non-foveal areas in S1, this suggests that within the lemniscal pathway foveal afferents converge less than the afferents for other rays.

Finding that a behaviorally important area of skin is over-represented in the central nervous system is not new[79]. But finding that the size of the somatosensory representation cannot be predicted by counting the number of fibers innervating that structure and multiplying by a constant "afferent scaling factor" is special[80]. This result is important because it suggests the mole PrV, and perhaps the rest of the lemniscal pathway, could be used to address questions about how the central over-representation of a foveal area of a sensory epithelium comes about.

Conclusion

The role of the PrV, compared to other regions of the STC is relatively understudied. The oversight is surprising given its vital role in organizing the somatosensory cortex. The lack of focus on the PrV might be due to the relatively unimpressive PrV in rodents compared to other trigeminal somatosensory specialists. There are many unresolved questions that could be addressed with comparative work. Just some include: Anatomically, what contributes to a hypertrophied PrV? Are there unique afferents? Is there less convergence? Within a nucleus, what contributes to the "foveal" area of higher resolution? Understanding these points will inform us on the forces that link the evolution of sensory surfaces and their central representations.

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This article finds tests the relationship between whisker innervation and cortical barrel area in six strains of mice breed for their different whisker patterns. The authors find that within a strain there is a linear relationship between the variables, but the slope of the relationship differs between strains. The existence of a linear relationship can be contrasted with case in the star-nosed mole.

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CANDIDATE
REVIEWS

Investigating Atypical Multisensory Processing in Individuals with Autism Spectrum Disorders

Justin Siemann

Blending information from multiple senses together into a perceptual Gestalt is necessary to understand the world. Autism spectrum disorders (ASD) are classically defined by a triad of symptoms, although sensory impairments have been consistently reported as well. Recently, there has been an increased interest in how individuals with ASD combine and integrate information from multiple senses. This article discusses current topics in ASD including neurobiology and sensory impairments with a focus on how atypical processing of multisensory information may be related to the symptoms found in ASD.

Keywords: *Autism, sensory, multisensory integration, temporal processing, audiovisual*

Functional connectivity:
A process used to describe how well brain regions are connected based on the temporal synchronization of the activity between these areas.

Autism Spectrum Disorders: Overview

Autism spectrum disorders are complex neurodevelopmental disorders characterized by repetitive or restricted behaviors, impairments in language and communication, and deficits in social or reciprocal behavior[1]. Currently, it is estimated that 1 in 88 individuals is diagnosed with an ASD and that males are 4 times more likely to be diagnosed than females[2]. Diagnoses of ASD normally occur by 3 years of age, but clinical symptoms may be present earlier in development. Based on the DSM-IV, Autism, Asperger's syndrome, and Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) are three disorders that fall within this spectrum. Individuals with autism can be further characterized as either classic or high functioning based on IQ measures. Asperger's syndrome and PDD-NOS differ from autism in that individuals with Asperger's do not have delays in communication and individuals with PDD-NOS either lack impairments in communication or repetitive behaviors or present mild forms of the triad of symptoms[3]. While DSM-IV classification is currently in use it should be recognized that the proposed draft of the DSM-V modifies the definition of ASD.

Neurobiological and Neurophysiological Findings in ASD

In order to investigate possible causes for the symptoms found in autism spectrum disorders, there have been a variety of studies that have focused on determining the neurobiology of ASD. Autism has been characterized by multiple descriptions of atypical structural and functional connections within the brain. A consistent finding is that children with autism tend to have larger brains and greater head circumferences that increase in the first few years of development, then reaches a plateau and growth slows later in life[4, 5]. It is thought that this overgrowth occurs during a critical time period in the development of the frontal and temporal lobes, but does not seem to impact the occipital lobes[6]. This thought stems from atypical morphology demonstrated by an increase in cerebral grey and white matter in the frontal and temporal regions[7]. Another developmental finding in ASD is altered minicolumnar structure. A cortical minicolumn is the fundamental unit in the cortex that is comprised of excitatory pyramidal cells surrounded by inhibitory interneurons[8]. Postmortem studies revealed that in the frontal and temporal lobes, there were increased numbers of minicolumns, pyramidal neurons in the minicolumnar structure, and the overall structure was narrower in individuals with

autism[8, 9]. More excitatory pyramidal neurons along with a greater number of narrower minicolumns could result in over activation of neurons, leading to local cortical connections more likely and long range connections less likely to develop[10]. These neurobiological and structural findings prompted the investigation of possible altered functional connections between specific brain regions in ASD. There have been numerous studies demonstrating a reduced functional connectivity between multiple brain regions in individuals with ASD[11-17]. It was shown that the functional connectivity between Broca and Wernicke's areas, two regions used for speech, was reduced in individuals with ASD compared to typically developed controls[18]. Based on impaired long range connections between regions used for speech processing, this finding provides neurological evidence for possible deficits in communication in ASD.

Autism Spectrum Disorders: Theories

In order to explain the neurobiology along with the symptoms found in this heterogeneous disorder a multitude of theories have been proposed. Weak central coherence, the temporal binding deficit hypothesis, the cortical underconnectivity theory, and an imbalance of excitation/inhibition signaling are relevant theories in explaining disturbances found in ASD[19].

Central coherence is based on the concept that individuals are capable of processing and combining information for a higher level of understanding[20]. The theory of weak central coherence (WCC) states that individuals with autism have impairments in integrating information from a local or detailed perspective to a more global concept[21]. If there is a preference for local over global processing, this may explain aspects of restricted interests and could cause the global meaning of social situations and communication to be impaired. Brock's temporal binding deficit hypothesis tried to explain weak central coherence by looking at potential timing deficits. This theory proposed that long range connections between brain regions may not be as temporally correlated as brain regions that are in closer proximity, which could result in intact local, but impaired global processing[22]. The cortical underconnectivity theory explained this concept in more detail, when functional magnetic resonance imaging (fMRI) studies demonstrated that long distance connections between brain regions were less functionally connected in individuals with autism[18, 23]. The cortical underconnectivity theory has been consistently used to describe atypical processing found in ASD. Just *et al* proposed this theory by using an fMRI sentence comprehension task to demonstrate atypical functional connectivity in individuals with ASD[18]. This theory compliments the previous two theories because it provides a possible reason for global information processing deficits since this type of processing requires the proper timing and integration of information from multiple brain regions[24]. A final explanation is a possible imbalance of the ratio of excitation/inhibition signaling in the brain. This is supported by post-mortem studies in ASD patients demonstrating an increased number of excitatory pyramidal cells[4, 10, 25]. While no one theory can explain all of the symptoms, there are aspects from all of these theories that may lend to greater insights in explaining the impairments that are seen in individuals with ASD.

Sensory Impairments in ASD

In addition to the three classical symptoms that impact individuals with autism, sensory abnormalities have been consistently observed and reported. Sensory dysfunction is not one of the classic symptoms of autism spectrum disorders, yet it is found to impact up to 85% of individuals with ASD[26]. Kanner first reported children with autism spectrum disorders to have sensory disruptions and to date there have been a number of studies demonstrating atypical sensitivity to sensory stimuli[26, 27]. Individuals can be hyper- or hyposensitive to a variety of different stimuli spanning multiple modalities[28, 29]. Using a visual stimulus as an example, individuals with ASD may immediately cover their eyes or stare at bright lights for long periods of time, depending on the individual's sensitivity to the stimulus. This dysfunction can be further classified as either sensory aversion or sensory seeking behavior. Depending on the type of behavior, individuals with autism spectrum disorders may be highly sensitive to or engaged in stimuli that may be considered mundane. Based on these observations, there have been several studies that have correlated sensory impairments with ASD severity[30-33]. Of all the sensory modalities impacted, dysfunctions in the visual and auditory domains have been studied most extensively. One reason for why this may be the case is that these modalities are needed for communication as well as understanding aspects of social behavior. A consistent finding is that individuals with ASD tend to perform well on visual tasks that require the processing of individual features[34, 35]. For example, individuals with ASD can excel on the Embedded Figures and Block Design tasks which are timed and require the identification of smaller objects within a larger figure or the recreation

of a detailed pattern using a set of blocks[20, 21], respectively. While there are reports of enhanced performance on tasks requiring the identification of specific features there have been observations demonstrating impaired global processing when these local features are needed to be combined. For instance, individuals with autism typically are less accurate in identifying biological motion, motion coherence, and visual form compared to controls[36-38]. An interesting finding from these studies however, was that while the high functioning autism group showed deficits in these areas, individuals with Asperger's syndrome performed as well as typically developed individuals, which speaks to the heterogeneity of ASD[37-38]. Similar to the findings in the visual domain, it is known that depending on the task, individuals with ASD perform atypically compared to controls in the auditory domain as well[39]. For example, it has been shown that individuals with ASD tend to excel on tasks that require pitch discrimination specifically of musical tones[40, 41].

Enhanced visual and auditory discrimination to detailed information, yet overall impaired global processing in these modalities are consistent findings in ASD. Communication and behaving in social contexts require a high level of global processing and if individuals with ASD have deficits in this type of processing this could result in information being lost or improperly understood. Besides the possible impaired processing, both communication and social behaviors require the appropriate combination of sensory information from multiple modalities. Based on these observations and studies describing unisensory dysfunction in ASD, there has been an increased focus in determining how multisensory integration may be impacted when sensory stimuli are combined.

Multisensory Integration and Temporal Processing

Multisensory integration can be described as the merging of sensory information from different modalities[42]. Studies have shown that the combination of information from multiple senses can produce behavioral enhancements that can increase accuracy and reduce reaction times[43]. Three principles: space, time, and effectiveness are used to describe multisensory integration[43]. Stimuli from different modalities that are presented in close temporal and/or spatial proximity can result in a maximal multisensory gain[44, 45]. The enhancement seen in behavior from the combination of multiple stimuli compared to one of the sensory stimuli presented alone describes multisensory gain[43]. One effective way to measure multisensory integration is by using cross modal illusions. These illusions can produce interesting behavioral responses based on how one modality affects another. For example, the sound induced flash illusion demonstrates that the auditory domain can alter the visual information that is perceived by individuals[46]. In this illusion when two auditory beeps are played in close temporal proximity to a single visual flash, most individuals perceive multiple visual flashes[46, 47]. Also, it was shown that as the presentation of the second beep is further delayed in time, participants are less likely to perceive the illusion[48]. Therefore, this illusion not only utilizes the temporal principle of multisensory integration, but also demonstrates the concept of a multisensory temporal binding window (TBW). The multisensory TBW can be described as the time interval in which two cross modal stimuli are bound together as a single unified perceived event[49].

It was demonstrated that individuals with ASD performed comparable to typically developed controls for the sound induced illusion, which illustrates that there is intact multisensory integration for simple cross modal stimuli[49]. However, using this illusion, it appears that children with ASD have an extended temporal binding window[50, 51]. Foss-Feig *et al* showed that individuals with ASD perceived this illusion more often when the presentations of the second beep were delayed for longer periods of time[51]. This study demonstrated a multisensory temporal binding window for individuals with ASD that was almost twice as wide as that of typically developed controls. An extended TBW and overall atypical temporal processing could impact communication and the proper understanding of social behaviors, both of which are known to be impaired in autism spectrum disorders. Importantly, it is known that the multisensory temporal binding windows tend to be wider for children in general and it is possible to narrow the TBW with the implementation of multisensory training paradigms[52, 53]. The plasticity of the TBW may allow for future developments of remediation tools for individuals with ASD.

In general, there have been a variety of studies supporting the findings of temporal deficits when individuals with ASD are asked to process multisensory information[54, 55]. Bebko *et al* simultaneously showed children identical videos on two monitors with the only difference being that one of the video tracks was temporally delayed[55]. This study demonstrated that while typically developed children preferentially looked at the videos that were synchronous, children with autism did not have a preference looking at either video, specifically when the stimuli were

speech related. This suggests that the ASD group may not have noticed that one of the videos was temporally out of synchrony, which was why there was no preferential looking for this group. If long range connections between brain regions in ASD are not as temporally correlated as in typically developed individuals then this could explain some of these results especially when more complex stimuli such as speech were used. Overall, these findings suggest that there are multisensory and temporal deficits in autism spectrum disorders, which will likely have a major impact on communication and speech comprehension.

Communication in ASD

The integration of visual and auditory information makes speech naturally, multisensory. With the known impairments of communication in autism, speech and audiovisual integration have been greatly studied topics. It has been shown that individuals with ASD tend to perform worse on audiovisual tasks that use human faces and voices, but perform typically on tasks using non-human stimuli[56]. One task that uses human speech stimuli to measure multisensory integration is the McGurk effect. In this illusion, participants see an individual's face and lips move to form the utterance of /ga/, while simultaneously hearing the utterance /ba/ which tends to cause participants to report the perception of hearing /da/[57]. The McGurk effect can be thought of as a measure of multisensory integration because the McGurk percept of /da/ represents a fusion of both visual and auditory information. Similar to the sound induced flash illusion the McGurk effect is temporally restricted[58]. Multiple studies have shown that children with ASD tend to perceive the McGurk illusion less often than typically developed individuals[59, 60]. This would suggest a possible impairment in multisensory integration since the fused percept was reported less often. An interesting finding however was that adolescents and adults with ASD perceived this illusion as often as typically developed individuals, which suggests that these impairments may improve over time[60, 61].

In addition to a decreased McGurk percept, studies have found that children with ASD tend to perform worse on the visual only condition[59]. This is demonstrated by an impaired ability to lip read, which would mean that individuals with ASD may not benefit from the added visual information especially in a noisy environment in order to understand speech appropriately[62, 63]. Smith *et al* presented individuals with sentences within varying levels of noise and participants were asked to identify specific words when the words were only heard or were seen and heard simultaneously[62]. This study demonstrated that individuals with ASD performed typically compared to controls on the auditory only condition, but were worse at identifying words when both audio and visual information were presented. Impaired multisensory and audiovisual integration in noisy environments, similar to those in the real world, could be one explanation for the communication impairments that are found in ASD. Recently, it has been shown that throughout typical development children can improve on speech in noise tasks[64]. In addition to this finding it was demonstrated that after training on a lip reading paradigm, individuals with ASD reported the McGurk percept as often as controls[59]. These findings provide evidence that this type of training may be another possible remediation tool for individuals with ASD, which may be the most beneficial if implemented earlier during development.

Overall, it has been shown that there are multiple levels of sensory dysfunction in autism spectrum disorders. Not only are there unisensory deficits, but multisensory impairments are also apparent. Atypical multisensory and temporal processing have been most evident in individuals with ASD especially as the stimuli become more complex and related to language. As studies have further characterized this sensory dysfunction, a few examples of possible remediation tools to improve multisensory integration, temporal processing, and speech comprehension have also been described. Multiple behavioral findings have described atypical unisensory and multisensory processing in ASD, which have been insightful in order to better understand this disorder.

Concluding Remarks

ASD is a heterogeneous neurodevelopmental disorder that impacts many aspects of an individual's life. Although multiple reports have described atypical structural and functional connections in ASD, there have not been many neurophysiological studies investigating deficits in multisensory integration and the brain regions that are potentially impacted. Russo *et al* published one of the first studies describing impairments in multisensory integration using EEG measures. This investigation demonstrated that multisensory integration within individuals with ASD was present specifically at later time intervals compared to typically developed individuals[65]. This further suggests that individuals with ASD are capable of integrating stimuli from

multiple modalities, yet temporal impairments are still evident. This study again supports the various findings of atypical multisensory and temporal processing. Based on functional imaging studies with typically developed individuals, one brain region that has been implicated in multisensory integration and temporal processing is the superior temporal sulcus[66-69]. This makes this region a potential area of interest in investigating multisensory impairments in ASD. Although there have been few neurophysiological studies describing multisensory and temporal processing in autism spectrum disorders, this allows for the opportunities of innovative research studies to be pursued.

The primary goal of this review was not only to give an overview of autism spectrum disorders and theories that have been used to explain these disorders, but also to specifically demonstrate how impairments in multisensory integration and temporal processing may relate to the common symptoms that characterize ASD. There have been numerous findings of atypical processing and sensitivity to unisensory stimuli spanning a variety of modalities. By studying how combinations of sensory stimuli are perceived, investigators have been able to further characterize the impairments in multisensory integration in individuals with ASD. Deficits in multisensory integration have led to findings of impaired temporal processing, which have generated an increased interest on how this could impact symptoms in ASD such as communication. This knowledge has generated behavioral studies focused on modulating audiovisual and speech stimuli. In addition to characterizing ASD behaviorally, there have been a variety of studies devoted to determining the neurobiology of ASD and how atypical development may impact connections and activity between brain regions in these disorders. A large number of neurophysiological studies have now characterized structural and functional impairments in individuals with ASD, but only a few have investigated multisensory processing. Additional studies that help to characterize atypical multisensory processing will not only improve our understanding of the impairments, but will also raise the possibility of developing better remediation tools for individuals with ASD in the future.

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Tactile Motion on the Glabrous Hand of Human and Non-Human Primates

Jeremy Winberry

Tactile motion is a complex perceptual experience that requires a nervous system built from the ground up to allow rapid processing of spatial and temporal sensory information. Tactile motion relies primarily on Meissner corpuscles (RA-1), although Merkel cell-neurite complexes (SA-1) and Pacini corpuscles (RA-2) also play roles. Primary afferent projections from these receptors transmit sensory information to the brain in large fast fibers, and with high fidelity. The organization of primary somatosensory cortex is optimized for location and receptor type. Receptive fields of neurons in SI adapt with motion in order to increase feature selectivity. Neurons responding to motion, direction, and orientation can all be found in SI. Complex stimulus features, such as motion velocity, are resolved by population coding. Higher cortical areas for motion processing, such as the human motion complex (hMT+), are probably multisensory. Vision, in particular, seems to share motion processing architecture with the tactile modality. As in vision, tactile motion illusions may shed light on the cortical processing of motion, particularly when paired with functional imaging techniques. Apparent motion and the tactile motion aftereffect are two such illusions discussed in this review.

Keywords: *Somatosensation, motion perception, primates, humans*

In order to discuss tactile motion, it is first necessary to reveal the relevant biological pathways in cutaneous motion perception. The real action in tactile motion perception begins at the receptor surface of the somatosensory system, namely the skin. When the skin is deformed by physical stimulation, specialized mechanoreceptive neurons called primary afferent neurons are depolarized. This is possible because the terminal ends of these neurons contain mechanotransducer channels. These channels are normally closed but open when flexed. The exact mechanisms for this opening are varied[1-2] and are not as well characterized as the mechanically gated channels of stereocilia in cochlear hair cells[3]. The induced currents from open channels can be recorded from the cell soma in the dorsal root ganglion[4]. Upon mechanical stimulation, these channels open, and cations, such as Na^+ and Ca^{2+}, rush into the terminal. If the inward rush of positive current is sufficient, then an action potential is produced. This basic transduction mechanism underlies the broad spectrum of mechanical somatosensation, including motion.

Mechanoreceptors of motion

The wide range of sensory percepts experienced is due to different morphologies and anatomic locations of primary afferent terminals. In order to discuss motion on the glabrous skin of the hand, it is necessary to consider at least these afferent terminals: Meissner corpuscles, Merkel cell neurite complexes, and Pacinian corpuscles. These different mechanoreceptors are typically classified by their rate of adaptation to a stimulus. The rapidly adapting (RA) mechanoreceptors are Meissner corpuscles (RA-I) and Pacinian corpuscles (RA-II), whereas the slowly adapting (SA) mechanoreceptor is the Merkel cell neurite complex (SA-I). A second class of slowly adapting mechanoreceptors, Ruffini corpuscles (SA-II), are not present in the glabrous hand of the primate and exist only in tiny numbers in the glabrous hand of humans[5]. SA-I afferents terminate at Merkel cells at the base of the epidermis, the outermost layer of skin[6]. These afferents are densely populated in the glabrous skin of the hand, and provide precise localization and pressure information. RA-I afferents terminate in Meissner corpuscles just below the epidermis[7], are layered with specialized Schwann cells, and respond well to slip, flutter and motion. RA-II afferents terminate at the base of the dermis in Pacinian corpuscles[8] and respond preferentially to vibration. Each of these mechanoreceptor types contributes to motion in a unique way.

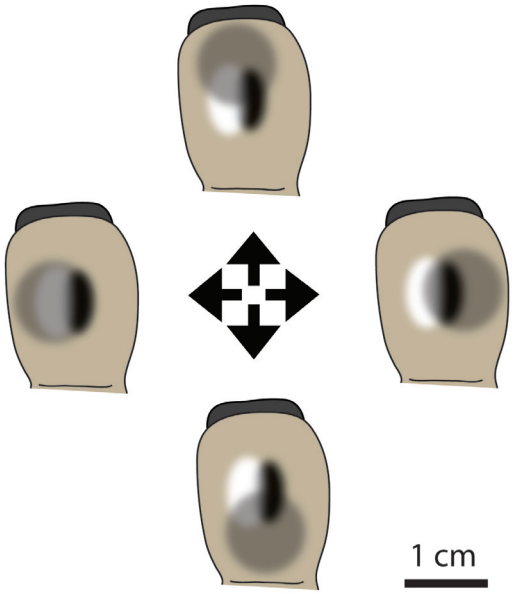

Figure 1: *Receptive fields as seen in Area 3b neurons.* On these four monkey fingertips, fixed excitatory (white), smaller fixed inhibitory (black) and larger lagged inhibitory (gray) receptive fields can be seen. The arrows in the center of the image signal the direction of a motion stimulus passing over the nearest fingerpad. The lagged inhibitory receptive field slides in the same direction as that of the motion stimulus, thereby modifying the overall receptive field. Note that these receptive fields are for illustration only and are not drawn to scale.

Dermatopic map:
A continuous repre-sentation of the somatic sensory surface, the skin. In this map, each fiber is beside the fiber that innervates the adjacent skin, and there are no breaks.

Somatotopic map:
A representation of body parts, some of which may be disconnected from the representation of adjacent skin areas. For example, the somatotopic map in primary somatosensory cortex has the thumb mapped next to the lower lip.

Modality:
A term that refers to the type of primary afferent, such as RA-1, SA-1, etc. Conservation of modality means that information from different primary afferent types stays segregated.

Mechanoreceptive afferent fibers can be individually recorded by microneurography[9-12]. This has allowed characterization of the output of the different mechanoreceptor types. This has also allowed direct measurement of receptive fields. Receptive field sizes for RA-I and SA-I fibers are 6.2mm and 4.8mm, respectively[13]. These receptive fields are not homogeneous, as they contain hotspots where some terminal branches are more sensitive than others[14]. For example, SA-I and RA-I fibers increase their firing rates linearly with indentation[15-16]. It was also discovered that making a second indentation in the skin just outside the receptive field produces a suppressive effect by means of relieving skin pressure in the receptive field[17].

Sensory transmission to the Central Nervous System

Mechanoreceptor afferent fibers are all large, myelinated A-beta fibers with conduction velocities between 36-73 m/s[18]. These properties are beneficial to rapid signal transmission to the central nervous system. Large-fibered mechanoreceptors project through the dorsal columns[19] of the spinal cord to the dorsal column nuclei. Dermatopy is preserved in the dorsal column, where sacral fibers are the most medial and cervical fibers are the most lateral[20-21]. These fibers rearrange before synapsing in the dorsal column nuclei, shifting from a dermatopic map to a somatotopic map[22]. The primate cuneate nucleus contains a complete somatotopic representation of the sensory surfaces of the hand[23].

Medial leminisal pathway. Under normal physiological conditions, dorsal column nuclei relay cells faithfully transmit the impulses of the primary afferent neurons[24-27].These cells project through the medial lemniscus to type I relay cells in the lateral division of the ventral posterior nucleus (VPN) of thalamus (Vc in humans). The VPN is also somatotopically organized[28-29]. The region of the VPN containing cutaneous afferents from the lemniscal tract projects to layers 4 and 3 of Areas 3b and 1 of somatosensory cortex[30].

Thalamus and SI cortex. Place and modality information are conserved from thalamus to Area 3b and 1[31], although signal transmission depends on vigilance. The amount of convergence from peripheral receptor to Area 3b is so restrained that Area 3b receptive field sizes are just 2-3 times the size of primary afferent receptive fields[32]. Dense microelectrode mapping demonstrates that there are somatotopic maps in areas 1 and 3b[33-34], demonstrating the

conservation of place information. The fingers representations in Area 1 and 3b point away from each other[35]. The hand representation can also be located histologically[36], or, in humans, anatomically[37-41] or by electrical cortical stimulation in waking humans[42-44]. These areas display a columnar structure[45], with different cortical columns representing different modalities, SA or RA[46].

All of the information so far illustrates that sensory information transduced by primary afferents in the skin is transmitted to cortex with high fidelity of place and modality information.

In some cases, primary afferent responses closely mimic psychophysical responses. For example, both primary afferent firing rates[15-16] and psychophysically perceived pressure[47] increase linearly with skin indentation. Another example is that RA-1 tactile thresholds match psychophysical thresholds[48-49]. Furthermore, a single impulse in a single RA-1 fiber from the fingerpad produces a tactile percept[12]. The cortex is essentially the bridge between stimulus transduction and tactile perception. Additional evidence linking cortical processing to this percept comes from finding BOLD response[50] and evoked potentials[51] from the same stimulus.

The rapidity and fidelity of tactile information, as noted above, allow for motion processing. If a finger is held on a surface without moving, spatial features of that surface are only weakly observed. However, even surface features a few microns tall can be perceived on a moving surface[52]. RA-1 afferents are thought to be responsible for this gain of function with movement[14,53]. This is especially true regarding slip of a smooth surface[54]. Because RA-1 afferents are silent when held motionless on a surface, only the SA-1 afferents collect useful information. When the surface is moved, the RA-1 and SA-1 afferents can respond to raised dots as small as 2-4 μm and 8 μm in height, respectively[52]. The detection of edges, as opposed to dots, is possible at sub-micron heights[55]. Interestingly, sensitivity does not change over a wide range of velocities (10-40 mm/s)[52]. However, optimal velocity ranges do differ depending on skin type and, presumably, receptor density. Essick et al.[56] showed that optimal velocities for motion to be perceived on the fingertip of humans ranged from 15 to 94 mm/s whereas for the proximal forearm the optimal velocities were 115-312 mm/s. Strokes of movement had to be 5.9 times longer on the forearm than on the fingertip in order to obtain the same sensitivity[56]. While increased force and velocity increase firing rate, the spatial pattern of firing does not change[57].

Cortical processing of motion

Primary somatosensory cortex processes basic sensory input to reveal complex features. One important cortical process is to use population coding. For example, localization discrimination thresholds on the fingertip can be as low as 0.38 mm for a 1.9 mm tactile probe[58]. Although there is overlap in the area that the probe depresses in such intervals, the population responses are different enough to discriminate. Significantly, this discrimination threshold is smaller than the receptive fields of single primary afferents. Other complex percepts, such as curvature, orientation, movement, and direction, also require population coding.

Receptive fields of Area 3b neurons have been described in detail[32,59-61]. 95% of these neurons have an excitatory field of about 24 mm^2 on the skin, with a range of 3-43 mm^2. However, about 5% of 3b cells in the same study had two or more excitatory receptive field regions. They also have an adjacent inhibitory field of about 18 mm^2, with a range of 1-47 mm^2. This configuration enhances feature contrast and preference. Remarkably, there is also a dynamic, delayed inhibitory field, whose position is not fixed, but rather biases in the direction of motion[32]. The 30 ms delay of this inhibitory field could serve to suppress minor features in the scanning direction on smooth surfaces and to emphasize novelty. At sufficient scanning speeds, it may even serve to confer directional

Orientation:
A cell is said to have orientation preference if its preferred stimulus passes through the receptive field at a specific angle, regardless of direction.

Motion:
A cell is said to have motion preference if its preferred stimulus moves through the receptive field at any angle and direction.

Direction:
A cell is said to have direction preference if its preferred stimulus moves through the receptive field in one direction, but not the opposite direction.

Intrinsic optical imaging: This functional imaging technique collects backscattered light from the cortex and detects metabolic activity as oxygen-deficient hemoglobin absorbs more light than oxygen-rich hemoglobin.

preference[61]. Thus, Area 3b is the first stage of processing in the somatosensory system known to process motion.

Cortical neurons in Areas 3b, 1, and 2 are known to be sensitive to direction (60%), motion (37%), and orientation (3%)[62]. These cells are evenly split between RA type and SA type receptive fields[63]. Motion cells are mostly located in Area 3b, whereas direction cells are mostly located in Areas 1 and 2. Most of this processing seems to occur in layer 3, as opposed to layer 4 where most thalamic projections terminate[64]. Direction variant cells have also been found[65-67]. These cells respond to stimulus movement toward or away from a specific spot in the receptive field, typically located over a joint. Although primary somatosensory cortex (SI) has a broad variety of motion sensitivity, the higher-level characteristics of motion, such as velocity, appear to be processed in higher cortical areas.

Motion processing is thought to follow the dorsal pathway, which is used to guide movements. A number of imaging studies have implicated inferior parietal lobe and the human motion complex (hMT+)[68-71], although it has been proposed that the medial superior temporal area (MST) rather than the middle temporal area (MT) processes tactile motion[72]. Area hMT+ is best known for its role in visual motion processing. However, thanks to functional lesion studies with repetitive Transcranial Magnetic Stimulation (rTMS), it also appears to be necessary for tactile motion speed perception[73-74]. In order to deduce whether the area is truly multisensory or not, several studies have looked at combined visual and tactile motion paradigms. Results have included findings of facilitation between modalities[75] as well as interference between modalities[76-77]. These studies imply that there are shared resources for visual and tactile motion, lending further support to the idea that hMT+ is a multisensory motion processing area.

Motion Illusions

One of the major goals of cortical studies of motion is to uncover the neural correlates of perception. One way to study this is to use illusions in order to dissociate perception from reality. If the cortical area follows the real stimulus instead of the perceptual experience, then the area is not implicated in the illusory processing. Since many complex cortical processes use population coding, studying such processes at the single neuron level is unfeasible. Imaging methods are much better suited to study population coding, as they can look at processing within or between areas. Intrinsic optical imaging and functional Magnetic Resonance Imaging (fMRI) have been used to study the funneling illusion, finding that Area 3b activation reflects the perceived location rather than the somatotopic location[78-80].

Several interesting tactile motion illusions exist and have been psychophysically characterized. One of these is apparent motion. Apparent motion is perceived motion created by sequential discrete tactile stimulations on spatially disparate skin locations. The spatial pattern of stimulation and type of movement (expanding, contracting, etc) have little effect on the saliency of the apparent motion, but the optimal range of inter-stimulus intervals varies with stimulus duration[81,82]. The cortical mechanisms of apparent motion are not fully understood, but methods are being developed to probe this question with intrinsic optical imaging and fMRI[83-84]. These techniques offer the wide field of view necessary to study population coding. However, they sacrifice considerable spatial resolution in doing so. One promising technology that could be applied to tactile motion imaging is voltage sensitive dye imaging[85]. This technique has already been applied to visual motion[86] and offers temporal resolution on the order of milliseconds. For a rapidly developing, complex stimulus such as motion, such a technique may be necessary to understand the mechanisms of cortical motion processing.

Another promising tactile motion illusion is the motion aftereffect (MAE). By generating a

continuous motion stimulus for a period of time (up to several minutes) and suddenly removing the stimulus, a tactile motion sensation in the other direction is produced[87-89]. This is very similar to the visual motion aftereffect. In fact, Konkle et al.[90] found that the aftereffect can transfer between tactile and visual modalities. This further suggests the existence of shared processing for motion in the two modalities. The tactile MAE is probably conferred through RA-1 afferents, as it is much more difficult to evoke in skin locations with a lower innervation density of these fibers[91]. There are now tactile MAE paradigms designed specifically to elicit the best responses from RA-1 afferents[92]. Interestingly, the tactile MAE is produced even with apparent motion across crossed digits[93]. This means that the tactile MAE reflects environmental space as opposed to tactile space. The cortical correlates of this illusion are unknown, but a recent fMRI investigation shows that only SI remains active during the illusion[94], suggesting a functional role in the illusion.

The biological study of tactile motion seems to be entering a new era. Whereas microneurography and single-unit electrophysiology still dominated the field at the turn of the 21st century, the pendulum is swinging towards studies of population dynamics in relatively large cortical areas. Imaging methods, such as voltage sensitive dye imaging, offer a larger scope on cortical processing. The presence of two robust tactile illusions may prove to be critical for dissociating sensory and perceptual processing.

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