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EDITORIAL

Vanderbilt Reviews Neuroscience, Volume 3: Insights into the Diseased Brain

Understanding pathologies associated with the human brain have always been an important component of scientific research in the neuroscience field. While brain function in normal or disease states is difficult to study, this year's qualifying class in the Neuroscience Program has endeavored to tackle it from diverse, often complementary angles. Psychiatric illnesses ranging from bipolar disorder, schizophrenia, stress and anxiety disorders, Huntington's disease, epilepsy and even neurodevelopmental autism spectrum disorders are reviewed, displaying the impressive breadth of research focus embodied in this group of students. Whether it involves studying gene-environment interactions, mitochondrial abnormalities, voltage gated sodium channels, or mutations and variations in receptors, transporters and hormones, this group of students share an underlying desire to advance our understanding of the maladies associated with human brain function. The third volume of the *Vanderbilt Reviews Neuroscience* comprises scientific reviews that give you a glimpse of the type of research frontiers that will impact our knowledge of the diseased human brain in the next several years.

The original intent of this journal was to provide a tangible reward for the considerable efforts given by students in their Neuroscience qualifying examinations. To extend upon this foundation, some of the high caliber reviews in this year's *VRN* have been selected for inclusion in major scientific journals. From Mark Wallace's initiatives along with our collaborations with Dr. Vivian Siegel, an external review committee comprising faculty scientists from different universities were selected to review the work of the students and deem its candidacy for publication in a top-tier journal. These reviews were chosen based on their knowledge of the background literature, novelty of research objectives, future research directions in the pertinent field and potential clinical implications of their research work.

While the ten stellar candidate reviews represent the gemstones of this year's *VRN*, we must also praise the Vanderbilt Brain Institute and its associated entities for their outstanding involvement in the local Nashville community. In this issue of the *VRN*, you will get a flavor of the various personal initiatives that students have undertaken to give back to their community. Some of these include helping local flood victims in Nashville after the flood of May 2010 and participating in various activities during the Brain Awareness month.

Overall the editors are very pleased with the quality of work complied in this issue of the *VRN.* We would like to first thank the incoming students for their timely cooperation. Next, we thank the Associate Editors, Maureen McHugo and Andrew Hardaway for their assistance and input in the compilation of this journal. Additionally, the *VRN* editors want to acknowledge the noteworthy, yet often unrecognized contributions by the Interdisciplinary Program Coordinator, Roz Johnson, and the VBI Administrative Assistant, Shirin Pulous. Many thanks go out to Chris Ciarleglio for founding this journal, creating all associated artwork, and for all the struggles that go along with the establishment of a new publication. Finally, we want to thank Mark Wallace for being an integral part of the Neuroscience community at Vanderbilt. His visionary leadership and collaborative initiates have helped shape the path and influence that the *VRN* has had within Vanderbilt University and the Nashville community.

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

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INSIDE

What to Expect

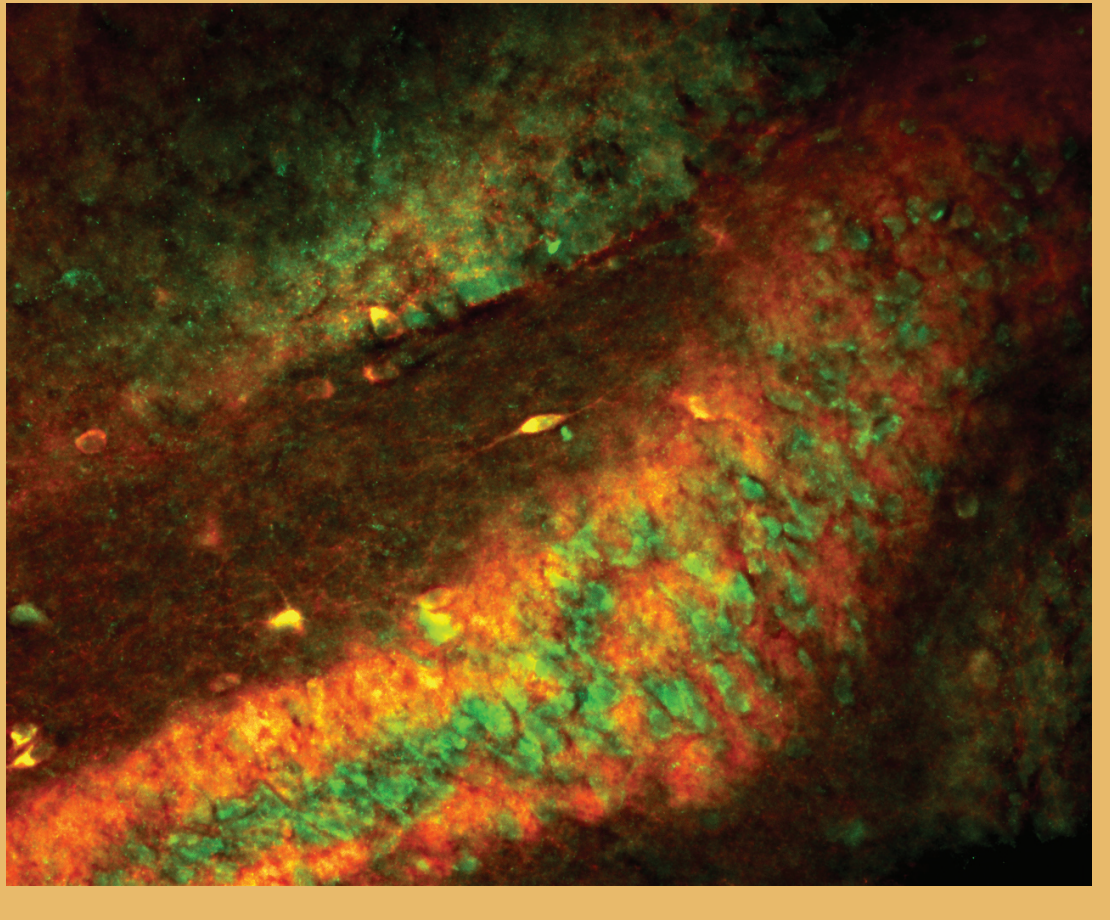
In this volume of Vanderbilt Reviews Neuroscience, we highlight a series of outstanding reviews from the qualifying class of 2010. These reviews cover a wide range of scientific endeavours, including the contribution of $GABA_A$ receptor mutations to epilepsy, mitochondrial abnormalities in bipolar disorder, and the clinical potential for neural lines of induced pluripotent stem cells. The variety of research presented by these talented students represents the Vanderbilt Brain Institute quite well, demonstrating the breadth of the interdisciplinary program.

As per tradition, we have also compiled a segment of mini-reviews of some of the groundbreaking research that has been published by Neuroscience Graduate Trainees in the past year. These publications cover a broad range of topics, including:

- Akt and schizophrenia
- Dopamine modulation of impulsivity
- Molecular response to stroke
- Unveiling the face of somatotopy
- Sensory processing in ASD
- Discovering modifiers of epilepsy
- Neuroprotective effects of spontaneous physical activity
- Detection of heavy metals in Huntington's Disease

On The Cover ...

Cells in ventral CA3 of the mouse hippocampus expressing proCCK (green) and GAD67 (red) are visualized via immunohistochemistry and fluorescence microscopy in a coronal section. GAD67 is consistently reported to be downregulated in post-mortem tissue from patients suffering from schizophrenia, while CCK+ GABAergic interneurons (yellow) appear to be one of the interneuron subtypes affected (Lewis *et al.*, 2005). Dysfunction of these cells has been associated with negative and cognitive symptoms in schizophrenic patients. However, since it is not possible to measure gene expression changes during the course of the disease in living patients, it is not known if this GAD67 expression deficit plays an active role in pathophysiology and behavior seen in the disorder or if it is a benign by-product. BAC-transgenic mice developed in Karoly Mirnics' Lab are being used to determine empirically whether a GAD67 gene expression deficit can cause physiological and behavioral disturbances and whether these alterations are dependent on the individual subtype(s) of interneurons affected (Garbett *et al.*, 2010).



-Martin J. Schmidt
Graduate Trainee,
Mirnics Lab
(See page 50
for review)

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Akt Linked to Model of Schizophrenia

Published in the June 2010 issue of PLOS Biology, Mike Suita, Sabrina Robertson, and other members of the Galli Lab provide a novel connection between protein kinase B/Akt and one of the classical hypotheses of schizophrenia: cortical hypodopaminergia. Using a conditional knockout of one of the primary members of the Akt-activating mTORC2 complex called rictor in the brain, they were able to assess the role of Akt signaling in maintaining cortical dopamine (DA) homeostasis. Consistent with rictor's obligatory role in the mTORC2 complex, these rictor null mice were found to have reduced levels of activated Akt in the prefrontal cortex. Behaviorally, these mice exhibited reduced sensorimotor gating as revealed by the prepulse inhibition (PPI) task, an endophenotype that is a hallmark of schizophrenic patients. Although there exist many prevailing hypotheses of the etiology of schizophrenia, one primary hypothesis is that changes in DA homeostasis in specific brain regions are required for the disorder. In testing this hypothesis, the authors found a significant increase and decrease in cortical tissue, but not extracelluar, norepinephrine (NE) and DA content respectively. These changes in monoamine levels were not due to presynaptic changes in DA neuron development or biosynthesis as rictor null mice do not show changes in DA neuron number or tyrosine hydroxylase protein. Since the presynaptic NE transporter (NET) is the primary regulator of cortical DA, they found that neuronal rictor KO mice have increased total and surface NET protein levels.

"Our studies provide a potential molecular mechanism linking Akt dysfunction to a schizophrenia-like phenotype and suggest the viability of targeting Akt phosphorylation and NET as pharmacotherapies for schizophrenia."

Additionally, synaptosomal preparations from these mice displayed increased uptake of [3H]DA and [3H]NE relative to floxed-only controls. In combination with their neurochemical data, these experiments suggest that neuronal rictor null mice show increased surface and total cortical NET protein that takes up extrasynaptic DA into cortical noradrenergic neurons which is then capable of conversion to NE. To test if the reductions in cortical DA are mediated by NET and are required for the deficits in PPI, they tested if a NET inhibitor nisoxetine could reverse these effects. Indeed, nisoxetine pretreatment significantly enhanced PPI and cortical DA in the rictor null strain, although the classical atypical antipsychotic clozapine was unable to rescue PPI in this strain. This is consistent with the requirement of Akt signaling in other antipsychotic medications, and might imply Akt as a convergent common pathway for schizophrenic medications. Although it is consistently ranked as one of the most costly and disabling neuropsychiatric disorders, schizophrenia treatments have not kept pace with other conditions such as mood disorders. These studies provide a critical link between a signaling molecule thought to be involved in the disease and one of its most prolific, yet controversial hypotheses. Since NET blockade was found to rescue the PPI deficits in the neuronal rictor strain, treatment of schizophrenic patients with NET inhibitors or mixed NET/DAT inhibitors provides a novel clinical avenue for study and treatment of the disease. Indeed, since improvement of the negative symptoms of schizophrenia like cognitive dysfunction (PPI) is the best indicator of patient outcome, NET/DAT inhibition might be a more effective target for future clinical studies.

Original Research Article:
Siuta MA, Robertson SD, Kocalis H, Saunders C, Gresch PJ, Khatri V, Shiota C, Kennedy JP, Lindsley CW, Daws LC, Polley DB, Veenstra-Vanderweele J, Stanwood GD, Magnuson MA, Niswender KD, Galli A. Dysregulation of the Norepinephrine Transporter Sustains Cortical Hypodopaminergia and Schizophrenia-like Behaviors in Neuronal Rictor Null Mice. *PLOS Biology* **8** (6) e1000393.

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Dopamine, a possible suspect for modulating human impulsivity

Buckholtz and colleagues (2010) wanted to explore the neural underpinnings of altered midbrain dopamine (DA) autoreceptor availability and its correlates with impulsivity trait and risk of psychiatric disorders. From pre-clinical findings, they found an important link between impulsivity and altered DA functioning, leading them to develop a neurobiological model for evaluating individual subject differences in human impulsivity. According to this model, they hypothesized that upon exposure to novel, salient or rewarding stimuli, highly impulsive individuals would be characterized by lower midbrain DA autoreceptor availability, leading to increased cell firing concomitant with potentiated DA release in terminal fields.

To test the efficacy of this model, 32 physically and psychiatrically healthy participants completed a Positron Emission Tomography (PET) scan using [18F] fallypride, a D2/D3 selective ligand that labels striatal and extrastriatal receptors. [18F] fallypride binds with high affinity to both presynaptic ("D2-short") and postsynaptic ("D2-long") D2-like receptors. Since DA receptor expression in the midbrain is primarily dominated by the D2-short receptor isoform, the variance in [18F] fallypride binding potential within the midbrain will be likely driven by individual differences in these D2-short autoreceptors. The participants were scanned in two sessions, one involving a placebo and the other involving an oral administration of 0.43 mg/kg d-amphetamine (AMPH). [18F] fallypride is known to be sensitive to endogenous DA release, particularly in the striatum, thus making it a useful ligand in both scan sessions, allowing assessment of both baseline receptor availability and individual differences in induced DA release.
Each participant also completed a Barratt Impulsiveness Scale (BIS-11) questionnaire which was correlated with the binding potential results from the PET scan.

Original Research Article:
Buckholtz JW, Treadway MT, Cowan RL, Woodward ND, Li R, Ansari MS, Baldwin RM, Schwartzman AN, Shelby ES, Smith CE, Kessler RM, Zald DH (2010). Dopaminergic network differences in human impulsivity. *Science* **329** (5991):532.

"Our findings suggest that dysregulation within ascending dopaminergic projection pathways subserving reward and motivation may produce deficits in impulse control, a critical feature of the psychopathological architecture underpinning substance abuse."

Buckholtz *et al.*, found that trait impulsivity scores were inversely correlated with D2/D3 autoreceptor availability in the substantia nigra/ventral tegmental area (SN/VTA) and positively correlated with the magnitude of the AMPH-induced DA release in the striatum. Since the midbrain DA autoreceptors inhibit DA release in terminal fields, the authors found that the SN/VTA D2/D3 binding potential was inversely correlated with striatal DA release after AMPH administration. They further probed the hypothesis that lower SN/VTA autoreceptor availability leads to impulsivity by enhancing DA release in the striatum. The results confirmed their hypothesis when they found that lower SN/VTA autoreceptor availability does in part lead to impulsivity by enhancing AMPH-induced DA release in the striatum. The authors went on to explore the nature of this enhanced striatal DA release to substance abuse risk. AMPH-induced striatal DA release and subject responses to AMPH (drug "wanting" ratings from the drug effects questionnaire) were measured. Interestingly, they found that increased DA release in the striatum predicted a stronger subjective desire for drug use after AMPH administration.

Since the present study showed that lower SN/VTA D2/D3 autoreceptor availability leads to higher DA release in the striatum correlating with higher trait impulsivity scores and stronger subjective desire for future drug use, the authors proposed a plausible link between impulsivity and drug dependence. Previous knowledge reporting stimulant exposure being a risk factor for future drug dependence and BIS scores predicting drug craving in substance dependent individuals, along with the findings of this study all converged on an important neurobiological link-- that human impulsiveness and drug abuse vulnerability were associated. Thus, Buckholtz and colleagues in this seminal paper provide evidence for possible associations between DA dysregulation and deficits in impulse control, an important feature of the psychopathology underlying substance abuse. Additionally, they provide a neurobiological model linking individual differences in DA network functioning to differences in human impulsivity.

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Exploring the Molecular Response to Ischemic Stroke

Stankowski JN, Zeigler SLH, Cohen EL, DeFranco DB, Cai J, McLaughlin B (2011). C-terminus of Heat Shock Cognate 70 Interacting Protein increases following stroke and impairs survival against acute oxidative stress. *Antioxid Redox Signal*. **14** (10): 1787-1801.

Stroke represents the third leading cause of death and the most debilitating neurological disorder in the United States. Ischemic stroke is the most predominant form of stroke, and is caused by an obstruction of blood flow to the brain, ultimately leading to cell death. The processes leading to cell death are multifarious and complex, but the Heat Shock Proteins (HSPs) and their associated interacting proteins are at the forefront of present research in the cellular response to stroke. HSPs are thought to contribute to neurological injury response through a coordinated system of chaperones and ubiquitin ligases, which recognize misfolded or damaged proteins and target them for degradation. Indeed, improper clearance of misfolded protein appears to be a hallmark of several neurological diseases, including ischemic stroke. Stankowski *et al*. investigated the role of CHIP, an HSP70 interactor, in ischemic injury models.

First, the investigators obtained post-mortem brain samples from individuals who suffered from a history of transient ischemic attacks (TIA), died from stroke, or controls. Their immunoblots for CHIP indicate that the protein is upregulated in TIA and stroke tissues, though not in controls, suggesting that the CHIP is expressed as part of the stroke response. They followed these human tissue trials with a series of in vitro experiments, finding that an oxygen glucose deprivation paradigm (stroke model) lead to neurite retraction in neural cultures, decreased expression of the main cellular antioxidant, and increased expression of FK2 antibody, a marker of ubiquitinated proteins. Furthermore, when they overexpressed CHIP (OE) in neuronal cell lines, they noted an increase in protein oxidation and ubiquitination in the presence of a proteasome inhibitor, hallmarks of cellular stress.

Previous studies suggested that CHIP played a protective role in the heart. Accordingly, Stankowski *et al*. investigated cell viability in their CHIP-OE neuronal lines. Surprisingly, CHIP-OE caused a 50% decline in cell viability. Conversely, knockdown of CHIP using small interfering RNAs (siRNA) lead to increased neuronal tolerance to oxidative stress. These results suggest an organ-specific role for CHIP in the response to oxidative stress, and overturn the hypothesis that CHIP represents a positive response factor in oxygen and glucose deprived brain tissue. Therefore, future therapeutic techniques may involve the knockdown, instead of enhancement of CHIP activity in the brain following ischemic stroke.

Unveiling the face of somatotopy – functional differences in the human S1 observed using high resolution fMRI

Stringer EA, Chen LM, Friedman RM, Gatenby C, Gore JC (2011). Differentiation of somatosensory cortices by high-resolution fMRI at 7T. *Neuroimage.* **54** (2) 1012-1020.

Stringer and colleagues in this paper investigated the ability of blood oxygen level dependent (BOLD) fMRI at ultra-high field strengths (7T) to differentiate functional activity in individual digits within area 3b and area 1 of the human somatosensory cortex. Previous studies in lower field strengths had reported findings with lower spatial resolution, where participant data were averaged to create a group map. Higher field strength magnets (7T and above) can theoretically provide greater sensitivity to BOLD signal as well as acquire data with higher spatial resolution within a short time window. In the current study, participants underwent a functional imaging scan while the glaborous skin surface on their nonadjacent digits were stimulated with air puffs at a frequency of 2Hz. Multiple runs were collected to map digit somatotopy and evaluate the reproducibility of single digit activations. Stringer *et al*., found that BOLD activations using their paradigm provided millimeter-scale detail of the S1 organization pattern in individual participants. They were able to show this reliably within each participant and across participants in digits 1 through 4 in both areas 3b and 1. Moreover, the authors were able to get reliable BOLD signal in a single participant scan in less than two and a half minutes. This is a potential advantage as scan durations can be a limiting factor in imaging patients who cannot be in an MRI for a long period of time. Another clinical implication of this study is noninvasive detection of small, yet localized cortical changes in individuals following spinal cord injury. Overall, the results of the present study by Stringer and colleagues make an important contribution to the field of imaging science and neuroscience as noninvasive 7T MRI is used to further our understanding of the fine grained functional map of the human somatosensory cortex.

Seeing with new eyes and hearing with new ears—a closer look at sensory processing differences in Autism Spectrum Disorders

Kwayke LD, Foss-Feig JH, Cascio CJ, Stone WL, Wallace MT (2010). Altered Auditory and Multisensory Temporal Processing in Autism Spectrum Disorders. *Front Integr Neurosci.* **129** (4)

Autism spectrum disorders (ASD) is typically characterized by deficits in social reciprocity, communication and behavioral flexibility. Recent diagnostic criteria have also included sensory abnormalities as central features of ASD. These abnormalities span individual senses in the visual, auditory, gustatory and tactile domains as well as information integrating different sensory modalities. In the present study, Kwakye and colleagues examined unisensory and multisensory temporal processing abilities in a single sample of children and adolescents with ASD by manipulating the extended temporal binding window for simple audio visual input. Temporal acuity in the auditory and visual domains was probed using temporal order judgment (TOJ) tasks to establish a baseline for auditory and visual temporal resolution. Following that, task irrelevant auditory signals were added to the visual TOJ task to assess the temporal nature of the multisensory binding processes. The authors found that there were no differences in visual temporal processing between children with ASD and those that were typically developing (TD). However, individual thresholds were higher for the auditory TOJ tasks in ASD children. Moreover, for multisensory processing tasks, children with ASD showed performance improvements over a wider range of temporal intervals than TD children. These improvements were manifested as higher accuracy and faster responses relative to the unisensory (visual-only) baseline condition. The authors thus provide evidence for an altered multisensory temporal binding window in children with ASD. An enlargement of this multisensory temporal binding window has important implications in the cognitive development of children. It can impair or delay language acquisition, as a child may not be able to correctly associate visual and auditory components of speech. Additionally, since successful social interaction integrates numerous visual and auditory cues, an altered temporal binding window early in life can create deficits for the development of more complex social abilities like empathy and reciprocity. The work done by Kwakye and colleagues provide important groundwork in advancing our understanding of the phenotypic sensory differences manifested in children with ASD.

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Clever Screen Uncovers Epilepsy Modifier

Over the course of the past decade, a multitude of human genes that lead to epilepsy have been identified. Epilepsy stems from abnormal excitability in neural circuits, manifest in seizures, yet the blanket term covers a variety of disorders, many of which show variable severity. Taken together, this suggests that a variety of genetic insults can potentially contribute to epilepsy. However, the majority of genetic screens have (intuitively) uncovered genes contributing to neural signaling, including those encoding the voltage-gated ion channels. In their work utilizing the *Scn2a*^{Q54} transgenic mouse, Jorge *et al*. uncover a strain-specific contribution of the potassium channel subunit Kv8.2 (*Kcnv2* gene) to the severity of seizures and ultimate survival of the transgenic mice. Importantly, these results identify Kv8.2 as an epilepsy modifier and cast a wider net for the contributions of all ion channels toward epileptogenesis.

This work began with the observation that the B6 and SJL strains of *Scn2a*^{Q54} transgenic mice showed differential phenotypes. The B6 resistant strain was less severe, with delayed seizure onset and increased survival. The group found that expression of Kcnv2 transcript was enriched in the hippocampus, the likely origin of seizures in *Scn2a*^{Q54} mice, but perhaps more importantly the SJL strain showed threefold greater expression of *Kcnv2* expression relative to B6. Next, a transgenic transfer approach indicated that the amino acid variation between the strains had little effect on epilepsy phenotype, but that increased expression of *Kcnv2* is sufficient to exacerbate epilepsy. Finally, the group found human significance for *Kcnv2*, uncovering two non-synonymous coding variants associated with two different types of epilepsy.

The findings by Kearney lab uncover the potassium channel subunit *Kcnv2* as an important genetic contributor to epilepsy. Kv2 channels contribute to the delayed rectifier K+ current in hippocampal pyramidal neurons, and function by dampening excitability following high frequency stimulation. The addition of the Kv8.2 subunit suppresses the delayed rectifier current, which could predispose neurons to increased excitability. Increased expression of *Kcnv2*, such as seen in the SJL mice, could decrease Kv2.1-mediated delayed rectifier potassium current, promoting excitability under repeated stimulation and a mechanism for the onset of seizures. This work highlights the interplay between ion channels in neuronal excitability, and harkens an expansion of ion channel screening to uncover new epilepsy genes and potentially new therapeutic targets.

Original Research Article:
Jorge BS, Campbell, CM, Miller AR, Rutter ED, Gurnett CA, Vanoye CG, George AL, Kearney JA (2011). Voltage-gated potassium channel KCNV2 (Kv8.2) contributes to epilepsy susceptibility. *PNAS* **108** (13): 5443-5448.

Neuroprotective genes correlate with spontaneous activity in rhesus monkeys

Mitchell AC, Aldridge G, Kohler S, Stanton G, Sullivan E, Garbett K, Faludi G, Mirnics K, Cameron JL, Greenough W (2010). Molecular correlates of spontaneous activity in non-human primates. *J Neural Transm.* **117** (12) 1353-1358.

In this Rapid Communication of the Journal of Neural Transmission, Amanda Mitchell and other members of the Mirnics Lab performed a study to assay the molecular correlates of spontaneous physical activity in rhesus monkeys. Briefly, the spontaneous activity, food intake, body weight and blood C reactive protein (CRP) were measured from a cohort of eleven female monkeys for two months. Brain RNA was harvested from these animals and used to detect changes in gene expression that correlate with any of these factors. The authors discovered that there is stable intrinsic variability in the spontaneous activity that doesn't correlate with changes in diet or food intake. In their expression study, they find that there is a direct correlation between the level of spontaneous activity and the level of two genes known to be involved in neuroprotection: BDNF and ARC. Conversely, they found an inverse correlation in the level of CRP and spontaneous activity/BDNF + ARC levels. This study suggests that CRP, which can respond to inflammatory or injurious processes, may mediate changes in gene expression in the brain that enhance neurodegeneration in diseases like ischemic stroke. Since CRP is known to modulate gene expression in other cell types, a compelling model is that the level of CRP is regulated by activity and the amount of peripheral CRP can influence transcriptional changes in the brain through the blood-brain barrier.

New Strategies for Cellular Heavy Metal Detection in the Fight Against Huntington's Disease

Kwayke GF, Li D, Bowman AB (2011). Novel high-throughput assay to assess cellular manganese levels in a striatal cell line model of Huntington's disease confirms a deficit in manganese accumulation. *Neurotoxicology.* In Press.

An emerging hypothesis is that environmental exposure to heavy metals through pesticides, dietary intake and occupational hazards may contribute to neurological disease risk. One of these metals, manganese (Mn), is receiving attention in the context of Huntington's Disease (HD) as mouse and cellular models of HD show a net decrease in Mn accumulation and decreased vulnerability to Mn-induced cytotoxicity. Existing technologies to assess cellular Mn levels have been expensive and time-consuming, so Gunnar Kwakye of the Bowman Lab developed a novel high-throughput assay called the Cellular Fura-2 Manganese Extraction Assay (CFMEA). Unlike traditional calcium (Ca2+) level assays, this assay relies on the ability of Mn to quench the fluorescence of fura-2 at an excitation wavelength where Ca2+ has no effect.

In this article of Neurotoxicology, the authors describe the optimal conditions for extraction of cellular Mn, assessed the experimental range of Mn detection using fura-2, and explored the effect of other metals under their experimental conditions. As a proof of concept, they explored the ability of CFMEA to detect changes in Mn levels in samples spike with increasing doses of Mn and found their assay sensitive to these perturbations. For their final experiment they used CFMEA to assay the ability of a wild type and mutant HD striatal cell lines to accumulate Mn. As in their previous study, the authors showed that HD striatal cell lines show a deficit in cellular Mn levels. The development of this assay now provides a high-throughput tool to understand the molecular mechanism of Mn homeostasis in HD, which is critical to understanding how environmental exposure may contribute to neurological disease.

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Surprise! Lateral PFC coordinates attentional networks.

Cognitive neuroscientists divide attention into stimulus-driven and goal-directed components, each with distinct behavioral and neural signatures. Goal-directed attention enables us to voluntarily direct attention toward a stimulus whereas stimulus-driven attention refers to the capture of attention by salient or unexpected stimuli. Previous research has shown that goal-directed attention relies on a dorsal network involving the frontal eye fields (FEF) and intraparietal sulcus (IPS). Conversely, ventral regions including the temporo-parietal junction (TPJ) and inferior frontal cortex support stimulus-driven attention. Asplund and colleagues examined how these networks might communicate.

The authors first developed a paradigm that isolates stimulus-driven attention in a way that does not recruit goal-directed networks. In the surprise-induced blindness task, participants searched for a target letter in a rapid serial visual presentation stream of distractor letters. On a small subset of trials, an unexpected ("surprise") distractor face stimulus was presented shortly before the target. The surprise distractor impairs one's ability to detect the target, but this effect persists only for 1-2 trials. Critically, this surprise distractor does not engage shifts in spatial attention nor does it require a response – both features that are known to recruit the dorsal attention network.

Original Research Article:
Asplund CL, Todd JJ, Snyder AP, Marois R (2010). A central role for the lateral prefrontal cortex in goal-directed and stimulus-driven attention. *Nat Neurosci* **13** (4): 507-512.

fMRI was used to probe the brain regions involved in this stimulus-driven attentional capture. As expected, Asplund and colleagues found that areas in the ventral attention network – TPJ and the inferior frontal junction (IFJ) – were more active when the surprise distractor captured attention compared to when it was successfully ignored. However, a more sensitive analysis revealed that the dorsal attention network also responded more to the surprise distractor when it captured attention. To better characterize the roles of dorsal and ventral networks, the authors analyzed the timecourse of the fMRI response to the surprise distractor within each network. This analysis showed a fast response within the TPJ and IFJ but a delayed response in the FEF and IPS, suggesting that the dorsal network is recruited too late to actually impair target detection. A separate experiment with a spatial surprise distractor confirmed that this timecourse difference was not due to hemodynamic latency differences between regions. Although expected, this finding does address how the stimulus-driven ventral network interacts with the goal-directed dorsal network.

The inferior frontal junction appears to coordinate activity in the dorsal and ventral networks, switching its pattern of activity depending on task demands. The authors first found evidence of this when they examined the timecourse of activity within the TPJ, IFJ, FEF and IPS during trials in which participants searched for a target without a surprise stimulus being presented. In contrast to its activity during surprise trials, IFJ activation on these surprise-free trials resembled the pattern exhibited by FEF and IPS. Asplund and colleagues next conducted a separate experiment designed to examine IFJ activity in a traditional goal-directed attention task. In this endogenous cueing task, participants are first presented with a central cue indicating on which side of the screen a target will subsequently be presented. The task demonstrates goal-directed attention because a valid cue that accurately predicts the target location enables participants to more rapidly detect an upcoming target compared to trials with an invalid cue. The data showed that as predicted, the pattern of IFJ activity closely corresponded to activity within the FEF and IPS on validly cued trials. This work nicely demonstrates how attentional networks integrate information to give rise to visual awareness.

Midbrain anatomy: not just for histologists anymore.

Eapen M, Zald DH, Gatenby JC, Ding Z, Gore JC. (2011). Using high-resolution MR imaging at 7T to evaluate the anatomy of the midbrain dopaminergic system. *American Journal of Neuroradiology*, **32** (4), 688–694.

Dysfunction of the midbrain dopamine system plays a key role in diseases such as Parkinson's and schizophrenia. Anatomical characterization of the substantia nigra and ventral tegmental area, from which dopaminergic neurons arise, may be helpful for understanding and tracking pathophysiology in these diseases. However, previous attempts to obtain detailed structural images of these regions at magnetic field strengths typically used in MRI have been generally unsuccessful due to poor signal to noise with increased image resolution. In this study, Eapen and colleagues used high resolution MRI at 7T in humans to achieve structural delineation of the SN and VTA. The authors used contrast-to-noise (which quantifies how separable different regions are) and volumetric measurements to determine the efficacy of two imaging sequences, GRASE and FFE. Both sequences provided excellent definition of the SN and VTA with comparable volumetrics but slightly greater contrast-to-noise for the GRASE sequence. Interesting, the authors found signal variability within the SN on FFE images, indicating that this sequence may be particularly promising for future work aimed at separating the pars reticulata and pars compacta within the SN. The volume tracing methods developed in this paper will also be useful for future clinical and basic science research as the results show individual differences in SN and VTA volume across subjects. Structural changes over time as indexed by volumetrics could be linked disease course and symptoms. These techniques will also be valuable for relating midbrain structural variability to personality traits and risk factors for disease. Finally, delineation of the SN and VTA can be used in functional MRI region of interest analyses.

Stopping isn't the same for all movement types.

Godlove DC, Garr AK, Woodman GF, Schall JD. (2011). Measurement of the extraocular spike potential during saccade countermanding. *Journal of Neurophysiology* (epub ahead of print).

The stop signal task (SST) is a well-developed paradigm that has been used to measure motor inhibition in humans and non-human primates with EEG, EMG and FMRI. It is thus a useful tool for understanding and characterizing inter-species and inter-methodology differences. There are two main variants: the saccadic SST, which measures one's ability to cancel a prepared eye movement, and the manual SST, which measures one's ability to cancel a prepared hand movement. Godlove and colleagues sought to determine whether a previously identified feature of spinal motor inhibition, subthreshold muscle activity, was present during oculomotor inhibition in the SST in the macaque monkey. To get around the difficulty of measuring oculomotor EMG the authors took advantage of a previously identified EEG component associated with eye movements, the saccadic spike potential. Two monkeys were trained in a saccadic SST in which they had to make saccades to targets on a majority of trials. On a subset of trials, a "stop signal" was presented at a variable time following trial onset, indicating that the monkey should withhold its response. Computational modeling was used to precisely characterize the motor inhibition on stop signal trials and relate this to the saccadic spike potential. Contrary to findings from the manual SST, Godlove and colleagues found no evidence for subthreshold muscle activity during successful inhibition of saccades. The authors attribute this result to the differences in the nature of the saccade and spinal motor systems. Unlike the actions initiated by the spinal motor system, saccades are ballistic in nature – once begun, the action runs to completion. Much of the work in understanding the behavioral characteristics and neural systems involved in the SST has been done by Vanderbilt researchers and this work nicely highlights the interdisciplinary and collaborative nature of the research done by VBI students.

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A Message from the Neuroscience Student Organization President

The Neuroscience Student Organization (NSO) has existed at Vanderbilt for nearly a decade, and the role of this organization continues to evolve. The purpose of the NSO is to improve the quality of life of the neuroscience graduate student. In the past, the NSO has organized activities ranging from student socials, community outreach, and to mock qualifying exams and has invited outside speakers to Vanderbilt. In continuing with these events, the past year has seen an expansion of some of the responsibilities of the NSO and a coincidental change in the structure of the NSO board of officers.

In previous years, a tremendous burden has fallen on NSO officers (particularly the retreat, outreach, and academic coordinators), on top of the demanding schedules required of every neuroscience graduate student. With this in mind, the NSO was restructured to include a retreat committee and an academic committee, allowing the work required of one individual to be shared by a group of three individuals. Opening a committee for the outreach coordinator position remains a goal for the upcoming elections.

Over the past two or three years the NSO has become increasingly involved in the planning and execution of the program's retreat, with this years retreat committee taking charge of planning the location, dates and agenda for the retreat. An effort is being made to dial back the feeling of being at a conference and improve the social aspects of the retreat, while still bringing in a high profile speaker, Thomas Insel, the director of The National Institute for Mental Health.

In community outreach efforts the NSO has consistently organized the annual Brain Blast, an event targeted towards kids, allowing them to see different model organisms, make brain hats, test their senses, and even hold a real human brain. This year, Brain Blast moved from the Adventure Science Center to Vanderbilt Children's Hospital, making it possible for patients to participate in this fun event as well. The event was a huge success thanks to our Outreach Coordinator, Teniel Ramikie and the time and resources of many generous individuals at Vanderbilt. This generosity also showed itself in 2010 when many people in the Nashville area lost everything in the May flood. Members of the NSO were able to organize relief efforts and help in the clean up following the flood, and it got us out of the lab too!

One of the largest undertakings of the NSO, and arguably the most important, is the job of the academic coordinator, now the academic committee. This group is charged with organizing and moderating mock qualifying exams for neuroscience students preparing for PhD candidacy. The academic committee sets up a separate mock qualifying committee for each student based on his/her real committee, usually pulling students from the labs of assigned committee members. This is an invaluable benefit to students as the mock qualifying exam takes some of the fear out of the real qualifying exam, although it often encourages stricter studying habits.

NSO students provide flood relief in May 2010.

As I stepped into the role of NSO president, I had an important goal in mind for the NSO, that being to encourage more student involvement. While we have made improvements and are working hard to provide social outings that will interest everyone, I have also seen how difficult it is to pull a neuroscientist away from the bench, a rig, or screens and screens of data. While encouraging hard work and a love of all things neuroscience, the NSO also strongly encourages you to get out and meet those people who will be your future collaborators. Become an active member of the NSO, get involved, eat free food, and make new friends!

Sincerely,

Pete Volbrecht
NSO President

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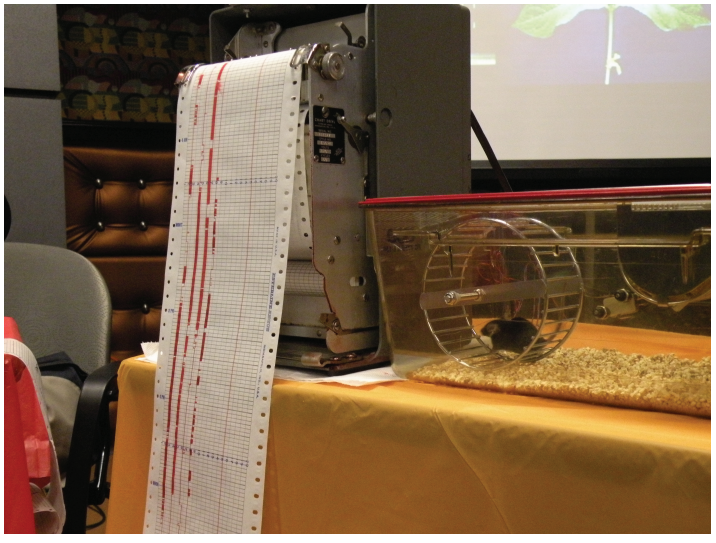
BRAIN BLAST 2011



Mice and real brains were on hand for neuroscience demonstrations at Brain Blast.

Brain Awareness Month 2011

The American poet, Robert Frost once said, "The brain is a wonderful organ; it starts working the moment you get up in the morning and does not stop until you get into the office." Though not the best of examples, Frost's facetious remark strangely brings to light the important role of scientists in our society.

Scientists have a uniquely dual role of guiding the helm of scientific discovery yet finding creative ways to present these complex discoveries in an accessible format to the general public. Such public outreach endeavors often allow us to not only share new and exciting information about the mysteries of science but also dispel innocently misguided affirmations regarding our field of interest.

Here at Vanderbilt Brain Institute, this principle of the scientist's dual role has become a staple in its training program of future neuroscientists. As graduate students, we have the gracious support of our administration to further encourage our public outreach interests. Such support is best exemplified by the success of our co-sponsored, annual Brain Blast event: an educational, family fun day that seeks to provide the Nashville community with stimulating opportunities to learn about the brain.

Brain Blast 2011 was met with an overwhelmingly enthusiastic response from both the neuroscience graduate student body, and the Vanderbilt neuroscience community. Collectively, we shared our passion for neuroscience with over 500 attendees from the Nashville community—one of the largest attendance in the history of the event, in spite of the inclement weather. Such an attendance demonstrates the strong public interest in neuroscience and further echoes the need for emerging scientists to actively engage the public. Through additional outreach activities, such as those taking place during the upcoming Brain Awareness Month 2012, or even by talking about our own research to members of our individual communities, we can continue to share with others a fact that even Frost, himself, had to admit—the brain is, indeed, a wonderful organ.

Sincerely,
Teniel S. Ramikie
Graduate Trainee, VBI

Hayley B. Clay as a neuron.

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A Note from the Director

It is a delight to see VRN continuing strong under its new editorial leadership, who has done another exceptional job in compiling our third volume of the journal. In previewing this year's edition, I am pleased to see the quality of the science encapsulated in this year's candidate reviews, and also to see the productivity of our trainees reflected in the articles selected for recognition in the "research highlights" and "in brief" sections of the journal.

The past academic year was another very successful one for our program, with fourteen of our trainees receiving their PhDs and thirteen new students entering the program. Although challenges abound in these difficult financial times, our program and the neuroscience research endeavor continue strong, and I am extremely optimistic about the future of neuroscience training and research at Vanderbilt. The combination of a world-class training faculty, exceptional resources and infrastructure, and a deep institutional commitment to the neurosciences bode well for the continued success of our program. Most importantly, our past and future success is ultimately the product of the creativity and hard work of our trainees. The work highlighted in the current issue of VRN gives me great confidence that we will continue to thrive as we move forward, and that we are well positioned to extend our international reputation as a leader in the realm of neuroscience discovery.

Yours in science,
Mark T. Wallace, Ph.D.

The Middle Tennessee Chapter For The Society For Neuroscience (MTNCSfN) Launches A New 'Slytherin Serpentine' Program

You might not know that the MTNCSfN is the oldest neuroscience organization at Vanderbilt University. Formed in the 1970s before there was either a PhD program in Neuroscience or the Brain Institute, this local chapter of the National Society for Neuroscience began to sponsor activities to unite neuroscientists in Middle Tennessee and to promote education and outreach at the local level. For many years the MTNCSfN has continued to support activities such as "Brain Blast" and other brain awareness events and also has nominated graduate students and postdoctoral fellows for travel awards to the national meeting every year. This year, however, the governing board of the chapter (Dr. Vivien Casagrande, President, Dr. Aaron Bowman, Secretary/Treasurer and Andrew Hardaway, Graduate Student Representative) decided to develop a more sustainable program for the chapter. SERPENT, which stands for "Summer Enrichment Research Program in Education and Neuroscience Training", was designed to provide a summer research internship for undergraduates from Middle TN area colleges and universities where research opportunities in neuroscience do not exist. The additional goal of this program is for these students to learn communication skills and develop educational materials to excite the next generation of young scientists. They will use this interactive program as they step into 5th and 6th grade classrooms to educate future "brainiacs" about cutting edge neuroscience research. Launching such an ambitious program, however, takes a significant financial investment, thus the chapter has been raising funds through a national SfN chapter grant, membership dues, and through contests such as the MTNCSfN Brain Lotto. The SERPENT program is now a member of the Vanderbilt Summer Science Academy (VSSA), which provides a well-structured summer program for a diverse set of summer research experiences. We are delighted to announce that Laurance Cain, a rising senior at Tennessee State University, was selected from amongst several excellent applicants, and is now interning in Bruce Carter's lab. The hope is that the SERPENT program will continue to sponsor more summer students in the future, create links between the chapter and local area universities to promote neuroscience research, and give the MTNCSfN a sustainable mechanism to educate future generations of neuroscientists. To ensure that future students like Laurance can participate in this unique program, the MTNCSfN invites you to contribute to SERPENT by renewing your chapter membership, donating to the chapter, and participating in future chapter fundraisers and social events. Please join us this October for our annual fall social and please visit the website below for more details about the SERPENT program and other MTNCSfN activities.
http://www.mtncsfn.org/

Vivien Casagrande, Ph.D.
President, MTNCSfN

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Program Update

The Neuroscience Graduate Program at Vanderbilt University under the leadership of Prof. Mark Wallace, Program Director will enter its thirteenth year of existence. For the 2011-2012 academic year, the graduate program will have 73 graduate students pursuing their doctoral degrees in Neuroscience. These students come from 23 states and 8 foreign counties. We have 95 training faculty committed to preparing our students for careers in teaching and research. Our students receive strong academic and research training from our outstanding training faculty.

Roz C. Johnson, B.B.A.
Interdisciplinary Program Coordinator

Nashville Gets A State-Of-The-Art Interactive Neuroscience Exhibit

In early 2010, the Silvio O. Conte Center for Neuroscience Research at Vanderbilt University, under the direction of Randy Blakely, Ph.D., secured stimulus funds from the American Recovery and Reinvestment Act (ARRA) to create a neuroscience-themed exhibit in Nashville, TN. Originally slated for the Adventure Science Center (ASC), the advisory committees of both the Conte Center and the ASC agreed that it was best suited elsewhere. By October of last year, the design process was underway for a set of interactive exhibits at Vanderbilt's own One Hundred Oaks campus, primary home of many of the Vanderbilt Medical Center's prestigious clinics. In addition to the new venue, the Conte Center enlisted the help of a new partner to manage the design of the project: Nashville's own *Anode, Inc*., which specializes in graphic design and interactive digital signage. Staying true to ARRA's mission of stimulating businesses in the local economy, Anode next secured the services of *1220 Exhibits, Inc*., another Nashville company and one of the preeminent exhibit fabricators in the country. Together with co-direction by Mark Wallace, Ph.D. and the Vanderbilt Brain Institute, the exhibit was designed, fabricated and installed at breakneck speed.

Upon entering Entrance A (south side) of the One Hundred Oaks clinic, visitors are greeted by five incredibly well-animated 52" reactive displays that highlight the neurological bases for control of movement, emotion, sensation, memory, and sleep. This exhibit, called *Inside the Mind*, is further accented by several neuron-shaped fabric sails that grace the ceiling and wall, enriching an area of the clinic that was once barren. Those who travel north down the long mall corridor, or those who enter at the popular Entrance D and turn left, will find the premiere exhibit at One Hundred Oaks: *Brain Matters*—an open and inviting lounge area with three 36" touch-screens allowing patrons to explore topics in brain chemistry, brain anatomy and function, and mental health. Finally, just north of *Brain Matters* is *Neuroscience Discovery*, an exhibit dedicated to the pioneering neuroscience research being done at Vanderbilt as well as the history of the field. Like *Brain Matters*, *Neuroscience Discovery* features two 52" interactive touch-screens, and like *Inside the Mind* it is accented by idealized neuron-shaped fabric sails which adorn the wall and ceiling. Together, it is our hope that this exhibit speaks clearly to the lay public and captures the attention of young people who might be interested in fulfilling careers in research or medicine.

In recognition of their outstanding contributions to this endeavor, the following individuals are acknowledged: Randy Blakely, Conte Director; Mark Wallace, Conte Co-Director and VBI Director; Mary Michael-Woolman, Conte Finance Director; Denise Malone, Assistant to Dr. Blakely; Bret Stoffer, Anode Executive Producer; Richard Bess, Anode VP Sales & Marketing; Adam Goleniewski, Anode Creative Director; Brian Altman, Anode Senior Animator; James Muspratt, Anode Graphic Designer; Chris Lee, Anode VP Technology; Isaac Meek, Anode Finance Manager; Jeremy Benson, Anode Customer Sales Support Specialist; Emily Davidson-Nemoy, Anode Traffic Manager; Brandon Vestal, Anode Project Manager; Eric Kremer, Anode Interactive Application Developer; Valerie King, 1220 Account Manager; John Knebel, 1220 Project Manager; Bob Owen, 1220 Lead Builder & Lead Installer; Stephen Stenhouse, OHO Facilities Manager; Steve Eastling, VUMC miracle-worker; Sean Farrell, Batten & Shaw Inc. Senior Project Manager, Cyril Stewart, VUMC Director of Facilities Planning; Bobby Otten, VUMC Facilities Planning Project Manager; Janice Smith, OHO Facilities Director; Donna Glassford, VUMC Director of Cultural Enrichment; Mike Smith, Gilbert McLaughlin Architect; Ed Hubbard, Neuroscience History Guru; the entirety of the Conte Supplement Advisory Board.

Chris Ciarleglio, Ph.D.
Assistant Director, Outreach & Education
Vanderbilt Brain Institute

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CANDIDATE REVIEWS

Mitochondrial Abnormalities in Bipolar Disorder

Hayley Boyd Clay

Abstract
Bipolar disorder (BPD) is a common and severe mental illness that affects 3% of the population[1]. Although its disease mechanisms are poorly understood, several lines of evidence indicate that BPD involves genetic and functional mitochondrial disturbances. These studies include genetic linkage and gene expression studies, biochemical studies, and *in vivo* neuroimaging. Animal models indicate that mitochondrial perturbation can by itself produce mood disorder-like phenotypes. Additionally, the mood stabilizers used to treat BPD exert effects on mitochondria, enhancing mitochondrial-related gene expression and the functionality of mitochondrial enzymes, protecting cells from mitochondria-mediated cell death. This review discusses mitochondrial physiology and its links to BPD and the mechanisms of action of the mood stabilizers used in the treatment of BPD.

Key Words: *mitochondria, bipolar disorder, energy metabolism, mtDNA, gene expression, valproic acid, lithium*

Introduction

Bipolar disorder (BPD) is a common, severe mental illness in which patients suffer episodes of mania and depression[A]. The clinical presentation of BPD can vary among patients, but a common finding in genetic, biochemical, and neuroimaging studies is evidence of mitochondrial dysfunction. As a high-energy tissue, the brain relies heavily on energy production through mitochondria-mediated oxidative phosphorylation (OXPHOS)[2-3]. The brain, then, is particularly vulnerable to alterations in mitochondrial function, and mitochondria-related mutations often result in brain pathology, such as blindness or seizure[4-5]. Slight or major alterations in mitochondrial function—whether through mutation, alterations in levels of the mitochondrial genome (mtDNA), or reduced enzyme function—may play an important part in the pathophysiology of BPD. Accordingly, the mechanisms of action of the mood stabilizers[B] used to treat BPD may compensate for mitochondrial deficits by improving OXPHOS capacity or preventing mitochondria-mediated apoptosis. This review discusses mitochondrial genetics and physiology, the evidence for mitochondrial involvement in bipolar disorder, and the effects of mood stabilizers on mitochondrial function.

Mitochondria and mtDNA

Mitochondria are intracellular organelles with prominent roles in energy production through OXPHOS, intracellular calcium (Ca^{2+}) buffering, and regulation of apoptosis. Mitochondria possess outer and inner membranes, which are separated by the intermembrane space. The innermost space, the matrix, contains enzymes and metabolites needed for the Krebs cycle. The inner membrane is folded into cristae and houses the enzyme complexes used in electron-transport chain (ETC)-mediated energy production. Each mitochondrion contains multiple copies of a 16.6-kB circular genome (mtDNA) that encodes 37 genes: two rRNAs, 22 tRNAs, and 13 ETC genes[4-6]. The remainder of the 1000-plus genes required for mitochondrial function is encoded in the nuclear genome (nDNA)[4-5].

Most cells contain hundreds of mitochondria, and therefore also contain hundreds or thousands of mtDNAs, though cellular content varies with tissue type[2-4,7-9]. Typically, more mitochondria and mtDNA are found in high-energy tissues such as muscle and brain[2-3]. Because human cells are polyploid for mtDNA, different mtDNA genotypes can exist within the same cell, a state termed heteroplasmy. When mutant and wild-type mtDNAs exist within the same cell, the cell only becomes symptomatic of mitochondrial dysfunction when the proportion of mutant mtDNAs passes a threshold, typically 80-90% of the cell's mtDNA[5-7]. During cell division, mitochondria distribute randomly to daughter cells; in the event of heteroplasmy, some daughter cells may receive a higher proportion of mutant mtDNAs, resulting in tissue-specific variations in mitochondrial genotype, or mosaicism (Figure 1)[5,7].

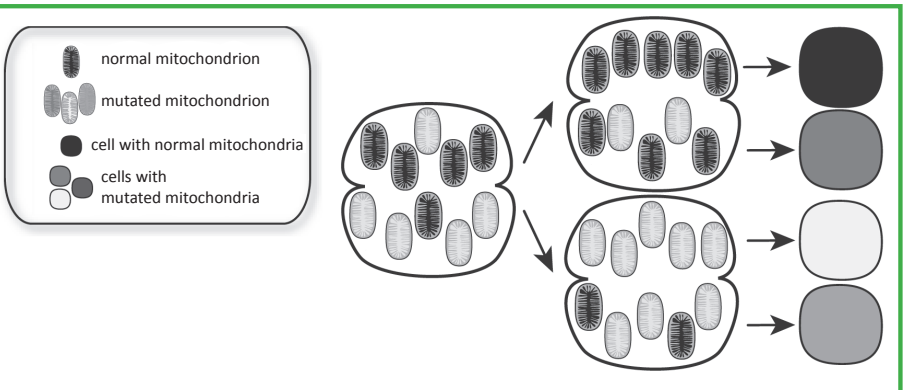

Figure 1. Heteroplasmy and mosaicism: Mitochondria containing both mutant and wild-type mtDNAs can divide such that some mitochondria become enriched for mutant mtDNAs. This is the basis for tissue-specific mitochondrial dysfunction.

MtDNA copy number regulation is important for the maintenance of mitochondrial function[10-14]. MtDNA depletion produces respiratory deficiencies inversely proportional to mtDNA levels[10-13]. MtDNA is packaged into protein-DNA complexes termed nucleoids, which house an average

[A] **Mania and Depression.** Affective states that define BPD. Mania involves elevated or irritable mood and hyperactivity, while depression is marked by sad mood and loss of pleasure, among other symptoms.
[B] **Mood Stabilizers.** Medications used to prevent episodes of mania and depression in BPD.

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of eight mtDNA molecules[15], mitochondrial transcription factor A (TFAM), mitochondrial DNA polymerase γ (POLG), the helicase Twinkle, and mitochondrial single-strand binding protein (mtSSBP)[6,16]. Each nucleoid protein is nDNA-encoded. These proteins work together in mtDNA replication, and mutation or knockdown of POLG or TFAM can result in mtDNA depletion and subsequent respiratory deficiency[10-13]. In fact, mice with neuronal TFAM knockout exhibited neurodegeneration and increased apoptosis concomitant with reductions in mtDNA14. Loss of mtDNA also correlated with decreased functioning of the ETC complexes that contain mtDNA-encoded subunits[14]. Overexpression of TFAM in mice increased expression of some mitochondrial genes, but did not increase respiratory capacity beyond that of control[17].

Mitochondrial physiology

Mitochondria are important for producing energy in the form of ATP. This process begins in the cytosol with glycolysis, which breaks down glucose into pyruvate. In the mitochondrial matrix, the Krebs cycle further metabolizes pyruvate to produce electron donors NADH+H+ and FADH. NADH+H+ donates electrons to complex I of the ETC (NADH dehydrogenase). FADH donates electrons to complex II (succinate dehydrogenase)[4]. Electrons move from complexes I and II to coenzyme Q, then to complex III (ubiquinol:cytochrome c oxidoreductase), cytochrome c, then complex IV (cytochrome c oxidase), where the electrons combine with O_2 to form H_2O[4,7]. As electrons move along the ETC, they release energy, which is coupled to the pumping of protons from the matrix to the intermembrane space. The protons create an electrochemical gradient across the inner membrane[4-5,7]. The energy stored in this gradient is released as protons pass through complex V (ATP synthase), back into the matrix. Complex V uses energy released from protons to condense ADP and inorganic phosphate (Pi) into ATP (Figure 2)[5,7].

ETC inhibition or dysfunction can lead to production of reactive oxygen species (ROS), which damage proteins, lipids, and nucleic acids. ROS form when electrons prematurely release from the ETC and combine with O_2 to produce the superoxide anion (O_2•-)[4]. Because mtDNA lies close to ROS production sites, mtDNA has a higher mutation rate than nDNA, acquiring both single nucleotide polymorphisms (SNPs) and large-scale deletions[4,7]. The most prevalent mtDNA deletion, the common deletion, is nearly 5 kB long[4]. Cells containing a very high percentage of deletion-type mtDNAs exhibit higher ROS production rates and increased susceptibility to apoptosis[18-19].

Mitochondria provide Ca^{2+} buffering for the cell. Stimulation of receptors such as those for N-methyl-D-aspartate (NMDA) or inositol triphosphate (IP_3) elevates cytosolic Ca^{2+} levels[20]. Mitochondria take up Ca^{2+} but release it slowly, preventing Ca^{2+} overload and excitotoxicity[20].

Mitochondria also participate in the regulation of apoptosis. Exposure to cytotoxic stimuli such as high Ca^{2+} levels, excess ROS, or loss of the mitochondrial membrane potential triggers the opening of the mitochondrial permeability transition pore (mPTP). While not yet fully characterized, the mPTP is thought to contain several mitochondrial proteins, including cyclophilin D, the voltage-dependent anion channel (VDAC), and the adenine nucleotide translocase (ANT)[21]. Opening of the mPTP results in loss of the mitochondrial membrane potential and release of pro-apoptosis proteins such as cytochrome c and procaspases[21]. Cytochrome c, apoptotic protease activating factor 1 (Apaf1), and ATP work together to activate procaspase-9. Caspase-9 activates procaspase-3, which, in turn, promotes disassembly of the cytoskeleton, degradation of the nuclear genome, and apoptotic cell death[22].

In addition to cytochrome c and procaspases, mitochondrial outer membranes house members of the Bcl-2 protein family, which are important in apoptosis regulation[21,23]. Pro-apoptosis Bcl-2 proteins such as Bax and Bid promote the opening of the mPTP, while anti-apoptosis proteins such as Bcl-2 and Bcl-xL bind and inhibit pro-apoptosis Bcl-2 proteins[21,23]. The balance of pro- and anti-apoptosis Bcl-2 family proteins determines cell survival. As such, o IN- expression of Bcl-2 enhar TER- mitochondrial respiration and resilience against Ca^{2+}-mediated stress, but Bcl-2 knockout results in premature death[24-25].

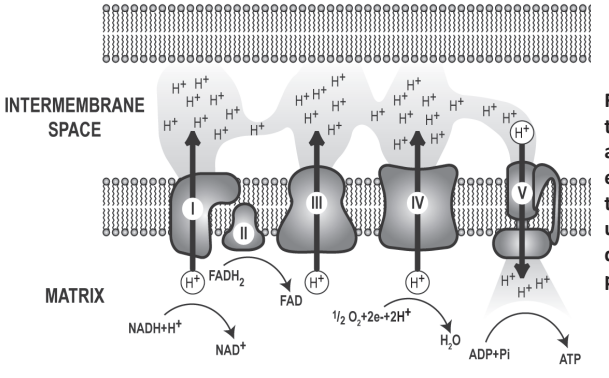

Figure 2. The electron transport chain (ETC): Electrons donated from NADH⁺H⁺ and FADH are passed along the five ETC complexes, releasing energy with each step. This energy is used to pump protons from the matrix to the intermembrane space. Complex V uses the energy stored in the electrochemical gradient across the inner mitochondrial membrane to produce ATP from ADP and P_i.

Mitochondrial disease

Mutations in mtDNA- or nDNA-encoded mitochondrial genes can result in mitochondrial disease. Because oocytes contain vastly more mitochondria than sperm, diseases stemming from mtDNA mutations or deletions exhibit a maternal inheritance pattern[5,7,26]. Alteration of OXPHOS genes impairs ETC function, and mutations in mitochondrial tRNAs inhibit mitochondrial protein synthesis. Also, POLG or TFAM mutations can prevent mtDNA replication, resulting in mitochondrial depletion syndrome[C,5-6]. Any of these alterations results in mitochondrial dysfunction and impaired energy production. Mitochondrial diseases commonly affect OXPHOS-reliant tissues such as muscle, brain, and pancreas, and involve symptoms like muscle weakness, blindness, seizure, and diabetes[4-6].

In addition to somatic symptoms, mitochondrial diseases are often comorbid with affective disorders and psychosis[5,27-30]. Inversely, BPD patients have increased rates of diabetes and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)[26,28]. As in BPD, mitochondrial diseases commonly do not appear until adulthood, and symptoms tend to worsen with time[1,7].

Mitochondrial abnormalities in BPD

Family studies have found a genetic contribution to BPD with an excess of maternal transmission, suggesting mitochondrial inheritance[31-35]. BPD-associated SNPs have been found across the mitochondrial genome[36-41], as well as in the nDNA-encoded complex I gene NDUFV2[42-44]. In addition to mitochondria-related SNPs, the common deletion may be more prevalent in the brains of BPD patients[45], though some have failed to replicate this finding[46].

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Expression of mitochondria-related genes is altered in BPD. Lymphoblastoid cell lines (BLCLs) derived from BPD patients had reduced mRNA expression of complex I and III subunits[42,47-48]. Glycolysis and OXPHOS genes were downregulated in the prefrontal cortex (PFC) of BPD patients[49-51]. Mitochondria-related gene expression was also reduced in the hippocampus[52], nucleus accumbens, and cerebellum in BPD[53]. In hippocampus, 43% of all downregulated genes in BPD were mitochondria-related[52].

Alterations in gene expression could stem from abnormal mitochondrial gene regulation. Lymphocytes from BPD patients were unable to respond normally to mitochondrial stress, failing to upregulate mitochondrial genes in response to low-glucose stress[54]. Altered mitochondrial mRNA levels were associated with reduced protein expression and mitochondrial enzyme activity, as well as increased oxidative damage in the PFC[55].

Mitochondrial Ca^{2+} buffering is also abnormal in BPD. Baseline Ca^{2+} levels were elevated in lymphocytes, platelets, and BLCLs of BPD patients[56-57]. Both platelets and BLCLs exhibited increased Ca^{2+} responses to stimulation[58-60], indicating increased mitochondrial Ca^{2+} content, possibly placing cells at a greater risk for apoptosis.

In addition to biochemical data, neuroimaging studies have provided *in vivo* evidence for abnormalities in the brains of BPD patients. In magnetic resonance spectroscopy (MRS) studies, patients exhibited reduced pH, ATP, and phosphocreatine (PCr) levels in the frontal cortex, indicating impairments in OXPHOS and a subsequent reduction in high-energy phosphate levels[27,28,61-62]. These changes may be state-dependent, as white matter PCr levels were found to inversely relate to depressive symptoms[63], and pH levels increased during mania compared to euthymia within individual patients[62].

Animal models have likewise provided links between mitochondrial abnormalities and psychiatric symptoms. Bcl-2 knockdown mice displayed increased anxiety-related behaviors[25]. Mice expressing neuron-specific POLG mutations exhibited increased startle amplitude and freezing, as well as altered circadian rhythms[64]. Treatment with the mood stabilizer lithium (Li) or electroconvulsive shock normalized the behaviors of POLG mutant mice[64-65]. The phenotypes seen in these animal models indicate that mitochondria-related mutations are sufficient to produce behaviors similar to those of BPD.

Mood stabilizers affect mitochondrial physiology and apoptosis

Valproic acid (VPA) and Li are two of the most common mood stabilizers used to treat BPD. The exact mechanisms of action of these drugs have not yet been fully elucidated, but both VPA and Li exert several different mitochondria-related effects.

Treatment with VPA or Li increased expression of mRNAs for nDNA- and mtDNA-encoded OXPHOS genes[66], and Li treatment prevented methamphetamine-induced reductions of complex IV expression[67]. Additionally, VPA and Li treatment increased expression of the transcription factors that regulate expression of mitochondria-related genes in both nDNA and mtDNA[66], opening the potential for VPA- or Li-mediated changes in mtDNA copy number.

VPA and Li treatment enhanced O_2 consumption and mitochondrial membrane potential in the SH-SY5Y neuroblastoma cell line[67]. In rats, VPA and Li exerted at least partial protection against amphetamine-mediated reductions in OXPHOS enzyme activities in the PFC, striatum, and hippocampus[67-68]. Additionally, Li treatment in postmortem human PFC enhanced the activities of OXPHOS complexes I-IV, indicating that mood stabilizers could directly promote mitochondrial function in human brain areas affected by BPD[69].

BPD patients often have elevated cytosolic Ca^{2+}, both at baseline and after stimulation. VPA and Li exert opposite effects on baseline Ca^{2+} levels[60,70], but both caused reduced Ca^{2+} spikes after stimulation with histamine or lysophosphatidic acid[60,70]. In mice, Li treatment reduced ROS levels and increased Ca^{2+} uptake capacity after Ca^{2+} exposure[71]. In addition, these mice contained mitochondria that were less swollen than those of non-Li controls[71].

VPA and Li reduced apoptosis rates after stressors such as serum deprivation and glutamate exposure[72-73]. Additionally, VPA and Li prevented cytochrome c release, caspase-3 activation, poly (ADP-ribose) polymerase cleavage, and excitotoxicity in ROS-exposed SH-SY5Ys[74-75]. VPA and Li not only prevented apoptosis, but also actively promoted survival through upregulation of the antioxidant enzyme glutathione-S-transferase, and increased protein and phosphorylation levels of Bcl-2[67,73-74,76-77]. VPA and Li also downregulated the pro-apoptosis genes Bax and p53[72,74]. Enhancing cellular resilience could translate to the reversal of gray matter loss that has been found in BPD[61,78]. Magnetic resonance imaging (MRI) studies of BPD patients on and off Li treatment showed that those taking Li had elevated gray matter levels in areas related to mood regulation, such as the hippocampus, anterior cingulate gyrus, and amygdala[76]. These data indicate that both VPA and Li improve mitochondrial health, and so may be a common mechanism of action toward mood stabilization.

Conclusion

Energy availability and mitochondrial health are crucial for proper brain function. In a tissue as OXPHOS-reliant as the brain, even a small perturbation in mitochondrial function could have detrimental effects. BPD is not a classical mitochondrial disease, as most BPD patients do not experience the symptoms of primary mitochondrial dysfunction. However, the pathophysiology of some or all BPD cases may receive significant contribution from mitochondrial abnormalities. The heterogeneity of mitochondria-related genetic susceptibility loci indicates that a number of abnormalities could result in similar symptoms, such as those seen in biochemical and spectroscopic studies. Animal models strengthen the link between mitochondrial dysfunction and psychiatric phenotype. VPA and Li at least partially reverse mitochondrial deficits, enhancing mitochondrial function and resilience by increasing OXPHOS-related gene expression and enzyme activity. The full scope of mitochondrial involvement in BPD is not yet known. Characterizing the exact contributions of mitochondrial dysfunction in BPD pathophysiology, however, will enhance overall understanding of the disorder and could help direct the development of more targeted and effective therapeutics.

[C] **Mitochondrial Depletion Syndrome**. A mitochondrial disease in which symptoms stem from a loss of mtDNA.

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Examining Potential Gene-Environment Interactions between the Parkinson's Disease-Associated Gene *parkin*, Manganese, and its Transporter DMT1

Sudipta Chakraborty

Abstract
Parkinson's disease (PD) is a neurodegenerative brain disorder that is marked by the loss of dopaminergic (DAergic) cells in the substantia nigra (SN). It is characterized by overall motor dysfunction, along with cognitive and emotional disturbances. Various treatment options currently exist to combat PD symptomatology, but are not able to directly target its pathogenesis due to a lack of knowledge concerning its etiology. In hopes of leading to therapy, many genes have been linked to PD, including *parkin*, which encodes for an E3 ubiquitin ligase involved in targeting proteins for proteasomal degradation. However, as the majority of PD is idiopathic[A], environmental factors must be taken into consideration as contributing to the disease as components of gene-environment interactions. One such environmental factor is manganese (Mn), an essential nutrient in our diet that can become toxic at high levels, leading to a condition known as "manganism" that resembles PD. Mn intoxication from excessive Mn exposure could arise from impairments in proper Mn transport mediated by the divalent metal transporter 1 (DMT1), or problems associated with intracellular Mn storage capabilities. Parallel findings (both clinical and pathological) between manganism and PD suggest common mechanisms that demand further examination. This review aims to shed light on the potentially interactive roles of parkin, Mn and DMT1 that may be involved in the pathogenesis of both PD and manganism.

Keywords: *Parkinson's disease, neurodegeneration, basal ganglia, parkin, ubiquitin-proteasome system, gene-environment interaction, manganese, DMT1, mitochondria, autophagy*

Parkin's Functional Connections to PD Pathophysiology

PD is one of the most common neurodegenerative disorders in the U.S. population, with a median age of onset around 60 years[1]. This disease affects more than 1% of the population over the age of 60 by causing preferential damage to the nigrostriatal[B] circuit of the brain. More specifically, distinct degeneration of DAergic neurons in the substantia nigra pars compacta (SNpc) is the prominent pathological hallmark of the disease, along with the presence of α-synuclein-rich[C] Lewy body inclusions. These features ultimately lead to motor dysfunction, with cardinal symptoms including bradykinesia (slowness in movement), tremors, rigidity, and postural instability[2]. Cognitive deficits and emotional and behavioral problems are also seen in diseased individuals. Later stages of the disease are often marked by appearance of a masked face, along with a forward-flexed posture, gait freezing, shuffling steps, and gastrointestinal issues[1]. Despite the myriad of known symptoms, the mechanisms behind this neurodegenerative disease are still unclear.

Although the majority of PD cases are sporadic in nature, about 10-20% of PD cases have a genetic basis. Many PD-associated genes have been identified in the literature, including *dj-1, pink1, parkin, NURR1, lrrk2, UCH-L1*, and *α-synuclein*[2]. This review will specifically focus on one of these genes, *parkin*, and its protein product, also called "parkin." This protein functions as an E3 ubiquitin ligase, a component of the ubiquitin-proteasome system (UPS) to target substrate proteins, along with itself, for proteasomal degradation[3]. It consists of 465 amino acids, and contains a ubiquitin-like domain that is responsible for substrate recognition, along with RING finger domains that interact with other components of the UPS[4]. Parkin expression in the brain is distributed within basal ganglia structures, including the SN and caudate-putamen, but also with some expression in the cerebellum as well[5]. Beside itself, parkin has many substrates, including the synaptic vesicle-associated protein CDCrel-1[3], α-synuclein[6], the α-synuclein-interacting protein synphilin-17, and the membrane receptor Pael-R[8]. Parkin has also recently been shown to form an E3 ligase complex with DJ-1 and PINK1, two other proteins implicated in PD[9]. Homozygous mutations found in parkin have been linked to an early-onset familial form of PD, with no presence of Lewy bodies[4]. Homozygous mutations in *parkin* result in altered intracellular localization, impaired substrate binding and enzymatic activity, both *in vitro* and *in vivo*. Consequently, a functional effect of mutations in this gene is an inability to degrade substrate proteins[10]. Parkin-/- mice show an increase in extracellular striatal dopamine (DA) concentration[11], while wildtype parkin seems to increase cell surface expression of the dopamine transporter (DAT) for increased DA reuptake *in vitro*[12]. *Parkin*-/- mice also show impairments in synaptic plasticity[13], as parkin seems to negatively regulate the strength and number of excitatory synapses[14]. Moreover, animal models expressing mutant *parkin* exhibit selective DAergic degeneration as well as hypokinetic deficits[15], as seen in PD cases. However, the mechanism behind these effects is still unclear and needs further investigation.

Gene-Environment Interaction: Manganese Neurotoxicity as a Cue

Despite the wealth of knowledge already gathered about the disease, mutations in parkin and other PD-associated genes still only partially explain a minority of PD cases. The possibility exists that a convergence between the individual effects of a genetic predisposition and exposure to

[A] **Idiopathic**: used to describe a disease in which the etiology is unknown.
[B] **Nigrostriatal:** major dopamine pathway that connects the substantia nigra with the striatum; involved in movement generation.
[C] **α-synuclein**: a soluble protein that can form abnormal aggregates (Lewy bodies) in neurons.

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an environment factor on the same neural pathways ultimately leads to PD. A twin study conducted in 1999 was one of the first to suggest that gene defects may not be the only causes of PD[16]. Furthermore, the recent Geoparkinson study found a significant association between pesticide use and PD[17]. Others have shown that exposure to the pesticide rotenone, a mitochondrial complex I inhibitor, causes selective degeneration of nigrostriatal DAergic cells[18]. Investigation into the effects of genetic risk factors on the ability of environmental factors to reproduce PD neuropathology has become more prevalent, as evidence for gene-environment interactions begins to expand. For example, in addition to causing selective DAergic cell loss, rotenone also induces oxidative modification of DJ-1, α-synuclein accumulation, and proteasomal impairment[19] (*DJ-1* and *α-synuclein* are also genetic risk factors for PD). However, support of a gene-environment interaction involving *parkin* in PD has been marginally examined. A recent study in Drosophila shows that *parkin* mutants exhibit a significantly shortened lifespan compared to control flies upon exposure to environmental pesticides[20]. With *parkin*'s prevalence as a major genetic risk factor for PD, further work must be done to look at its interactions with environmental factors that may be causing the pathophysiology seen in patients harboring a *parkin* mutation. Temporal parallels between PD and manganism further support a gene-environment hypothesis, where environmental exposure to manganese (Mn) and a juvenile, familial form of PD due to *parkin* mutations can both be found in younger individuals[21]. Thus, further work is necessary to assess whether Mn exposure is an added risk for individuals harboring polymorphisms in the *parkin* gene.

Consequently, environmental PD risk factors now include heavy metals. One such metal is Mn, a vital trace element that is necessary for proper metabolic function and detoxification of superoxide free radicals. Mn ions are important cofactors for several enzymes, including but not limited to glutamine synthetase, arginase, and superoxide dismutase (SOD)[22]. Mn also has a significant role in proper immune function, bone growth, digestion, reproduction, and defense against free radicals[23]. Despite being an abundant and essential micronutrient, rare Mn deficiency can result in abnormal bone growth, birth defects, impaired macromolecular metabolism, and seizures[23,24]. The essentiality of Mn, however, is mirrored by neurotoxicity that can develop from excess Mn exposure. Outside of dietary Mn intake, environmental sources of exposure can include drinking water (groundwater)[25], pesticides[26], and airborne exposure upon combustion of the fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT)[27]. However, excessive environmental exposure to Mn can also occur through a variety of occupations, including welders, steel miners, smelters, and other industrial workers[28]. Excessive exposure to Mn can result in an irreversible condition known as "manganism," which involves neurological disturbances that highly resemble PD. This disorder is marked by progressive bradykinesia, gait disturbances, fixed facial expression, and slurred speech[29]. Its pathology includes increased Mn concentrations in the basal ganglia, including the caudate-putamen, SN, subthalamic nuclei, and the globus pallidus[30,31]. Unlike PD, damage from Mn intoxication primarily occurs downstream of the nigrostriatal pathway, focusing on the globus pallidus output target. Interestingly, similar to *parkin* mutants, it is rare to see Lewy bodies in the SN of individuals suffering from manganism[24]. Mn has been shown to promote cytotoxicity in dopamine- producing cells[32], along with affecting neurite length and integrity within the basal ganglia[33]. Mitochondrial dysfunction is also generated upon Mn exposure in a concentration-dependent manner, with depletion of ATP and an increase in reactive oxygen species[34]. Furthermore, Mn also impairs proper astrocytic function, consequently hindering their buffering roles in protecting the more sensitive neurons from Mn toxicity[35]. Aside from affecting the same neural substrates damaged by PD[36], Mn has also been identified as a potential component of a gene-environment interaction. Few studies have begun to look at this connection[37], with one study finding that α-synuclein may interact with Mn to result in increased Mn toxicity[38]. Moreover, in the only study thus far to specifically examine a *parkin*-Mn interaction, Mn was found to increase parkin protein levels specifically in DAergic cells. Parkin also conferred protection from Mn-induced DAergic cell death *in vitro*, and was selectively redistributed to the perinuclear region in DAergic cells upon Mn exposure[39]. However, it still remains unknown how parkin is able to selectively protect DAergic cells, and how Mn is able to alter this ability.

Consequences Of An Impaired Manganese Transport Profile

DMT1-Mediated Import

A potential mechanism for the neurodegeneration evident in *parkin*-linked PD cases could involve disrupted Mn homeostasis, implicating problems with Mn transport and/or storage capabilities within the cell. The delicate balancing act between Mn's necessity for proper physiological processes and its paralleled toxicity calls for tight regulation to maintain proper homeostasis. Mn can cross the blood brain barrier through a variety of processes[40], but the identity of a single and definite Mn transporter remains a mystery. A well-researched transporter prominently involved in Mn regulation is the divalent metal transporter 1 (DMT1). DMT1 is a member of the natural resistance-associated macrophage protein (NRAMP) family and was formerly known as the divalent cation transporter (DCT)[41], in which a missense mutation in Belgrade rats or microcytic mice was found to impair proper iron (Fe) uptake[42,43]. At the cellular level, DMT1 seems to localize to the membrane. In tissue, it is ubiquitously expressed, but shows most prominent expression in the proximal intestine, kidney and brain[41]. Light and electron microscopy has also found DMT1 expression in glial cell bodies of the neocortex, subcortical white matter, and the hippocampus[44], while immunocytochemistry has found dense DMT1 staining in the caudate, putamen, and substantia nigra pars reticulata (SNpr) of the monkey basal ganglia[45]. The commonality in neural substrates and transporter expression between manganism and PD requires further examination of potential mechanisms surrounding DMT1's interactions.

DMT1 was given its name due to its ability to non-specifically transport various divalent metal ions into the cell. The transporter's specific connection to Mn has come from studies that have looked at the effects of Mn exposure on the expression of DMT1. For example, Mn-exposed rat pups are found to have increased levels of Mn *in vivo*, as well as increases in the protein expression of DMT1 throughout the brain[46]. Similarly, increased Mn uptake is evident upon impaired cellular iron status in astrocytic cultures, corresponding with enhanced DMT1 protein expression in these cultures[47]. These data provide evidence that DMT1 is likely a Mn transporter.

DMT1's possible role in a gene-environment interaction in PD emerges from its functional transport of an environmental toxin like Mn into cells. The transporter's connection to PD genetics has only recently been investigated. The motivation behind this investigation comes from the existence of an alternatively spliced DMT1B isoform that could be affected by post-translational modifications through the ubiquitin-proteasome pathway. Keeping in mind that parkin is a known E3 ligase in this pathway, overexpression of parkin in a neuroblastoma cell line lead to decreased DMT1B isoform levels and increased Mn toxicity. Additionally, immunoprecipitation and immunofluorescent studies revealed co-localization between this isoform and parkin in cells transfected with wild-type *parkin*[48]. This study serves as just an initial *in vitro* examination of parkin's modulation of DMT1

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via ubiquitination (see Figure 1A). Furthermore, recent evidence has shown that in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, expression of DMT1 increases significantly in treated animals compared to untreated animals. Treated animals also exhibit increased Fe accumulation, increased oxidative stress, and overall DAergic neurodegeneration in the SN. Moreover, the mutation in the DMT1 gene previously shown to impair Fe transport actually confers protection against MPTP and 6-hydroxydopamine-induced toxicity[49]. Thus, higher expression of DMT1 in the basal ganglia could potentially explain this area's particular sensitivity to Mn toxicity, as well as its vulnerability to the specific neuronal damage done in PD. Further work is necessary to assess if and how this mechanism is affected *in vivo* upon exposure to high levels of Mn. In addition, investigating how high exposure affects Mn transport kinetics in *parkin*-linked PD models could help explain the variations seen among these cases.

Intracellular Mn Storage

Outside of its import process, another component of proper Mn homeostasis is non-toxic storage of Mn within the cell. Despite its necessity for the actions of mitochondrial antioxidant enzymes (Mn-SOD)50, a possible mechanism for the neuronal death seen in *parkin*¬-associated PD could involve improper storage issues that promote toxic Mn accumulation within mitochondria[51]. Chronic Mn treatment leads to Mn increase specifically in the mitochondria of neurons and astrocytes[52]. Mn-induced neurotoxicity notoriously shows signs of increased intramitochondrial oxidative stress, increased release of cytochrome c, and overall mitochondrial dysfunction, similar to PD cases. Striatal neurons show dose-dependent decreases in the mitochondrial membrane potential and Complex II activity upon Mn exposure[53]. This decline in proper mitochondrial function is similar to what is seen in PD, or in PD models induced by poisons like MPTP and rotenone that both inhibit mitochondrial complexes[54].

A direct connection between *parkin* and mitochondrial dysfunction that could be relevant to this toxicity has been recently illuminated. In addition to its role as an E3 ligase involved in the proteasomal degradation pathway, recent evidence has identified *parkin*'s involvement in the autophagy of damaged mitochondria. Initial findings show that *parkin* and PINK1 (a mitochondrial-targeted kinase) interact to maintain mitochondrial integrity within DAergic neurons, and that functions downstream of PINK1 in this pathway[55]. Subsequent studies found that the PINK1/*parkin* pathway in Drosophila actually promotes fission and inhibits fusion of mitochondria[56]. More recent evidence has found that *parkin* translocation to damaged mitochondria with lowered mitochondrial membrane potential is dependent on PINK1 expression, followed by the aggregation of these mitochondria into the perinuclear region for autophagic elimination[57,58]. These studies have expanded on *parkin*'s sole duty as an E3 ligase, now adding regulation of mitochondrial trafficking to its resume. Such remarkable results signify that PD pathophysiology could arise from ineffective clearance of defective mitochondria due to mutations in *parkin* or *pink1*, ultimately resulting in neurodegeneration. Furthermore, this neurodegeneration could also be linked to increased DMT1-mediated Mn import in the basal ganglia due to higher transporter expression, with increased levels of Mn entering DAergic neurons and increasing ROS production within accumulated, damaged mitochondria (see Figure 1B).

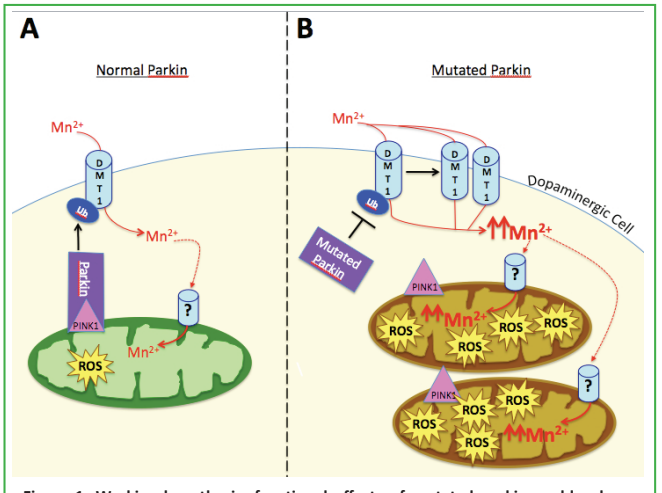

Figure 1. Working hypothesis: functional effects of mutated parkin could enhance Mn(II) toxicity to induce DAergic cell death in the basal ganglia. (A) Normal function of parkin could involve ubiquitination of DMT1 to regulate surface expression levels, controlling how much Mn(II) can enter the cell and, subsequently, the mitochondria to produce ROS. Additionally, interaction with mitochondrial PINK1 can promote mitophagy to reduce the number of damaged mitochondria. (B) A mutation in parkin could result in a protein that can no longer properly ubiquitinate DMT1, allowing for increased Mn(II) entry into the cell, and increased intramitochondrial Mn levels to produce a higher amount of ROS. Moreover, the mutated protein can no longer participate in mitophagy to remove Mn-intoxicated, damaged mitochondria.

Conclusion

Increased human life expectancy will soon designate the elderly as a growing proportion of the population, and a concordant rise in the number of PD cases will become an even more pressing public health concern. However, the signature selectivity of cell loss in the SN, marked by mitochondrial dysfunction and increased oxidative stress at the cellular level, remains an enigma. Nevertheless, the commonality in pathology, the neural substrates affected, and symptomatology shared by both PD and manganism suggests an interaction between genetics and environmental factors to precipitate these disorders. Based on the evidence presented in this review, future studies could focus on gene-environment interactions between Mn, parkin and DMT1. Increased oxidative stress and cell death in parkin mutants exposed to Mn could result from a higher degree of net intracellular Mn levels within aggregated, damaged mitochondria. Subsequently, increased intracellular Mn levels can react with dopamine via Fenton's reaction to create additional oxidative stress[59-61], further enhancing DAergic neurodegeneration, as seen in PD. It is also possible that the mitochondria themselves in DAergic neurons could be selectively vulnerable to environmental toxins like Mn. Thus, a multi-faceted, complex story emerges that could potentially facilitate the study of mechanisms to help point us towards novel therapeutic strategies.

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The Contribution of Voltage-Gated Sodium Channels to Inherited Epilepsies

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Abstract
Epilepsy is a common neurological disorder affecting approximately 3 million people in the United States. Epilepsies with unknown origin are attributed to genetic mutations with complex inheritance. Voltage-gated sodium channels are responsible for the initiation and propagation of action potentials and regulate neuronal excitability. Over 700 mutations have been identified in voltage-gated sodium channel genes *SCN1A* and *SCN2A* in human epilepsies, including Genetic (Generalized) Epilepsy with Febrile Seizures Plus (GEFS+) and Dravet Syndrome (or Severe Myoclonic Epilepsy of Infancy). Affected family members with the same mutation display variability in clinical severity of the disease. This suggests that additional genes modify the effect of the primary mutation, resulting in variable clinical presentation. Several mouse models have been generated to study the *in vivo* effects of genetic epilepsies. Many of the seizure phenotypes depend upon the genetic background of the mouse mutant strain, suggestive of genetic modifiers in epilepsy. Several neuronal ion channels have been identified as epilepsy modifier genes in mouse seizure models. Generation of mice with multiple neuronal ion channel mutations has resulted in more severe or ameliorated seizure phenotypes. In support of human epilepsy modifiers, mutations have also been identified in human orthologs of mouse epilepsy modifier genes. Identification of modifier genes that improve or exacerbate epilepsy may increase the understanding of the molecular events of epileptogenesis, advance molecular diagnostic capabilities and identify novel therapeutic targets for improved treatment of human patients.

Keywords: *Epilepsy; Genetics; Modifier Genes; Mouse Models; Voltage-Gated Sodium Channels*

Introduction

Epilepsy currently affects approximately 3 million Americans of all ages and 1% of the worldwide population[1]. Two-thirds of patients diagnosed with epilepsy have no known cause for their disease and in over 30% of patients, seizures cannot be controlled with currently available anti-epileptic drugs[2]. During the past 15 years, extensive research has identified genes that contribute to monogenic epilepsy. These mutations have been identified in genes encoding nicotinic acetylcholine receptors, GABA receptors, chloride, calcium, and voltage-gated potassium and sodium channels[3]. In contrast, less progress has been made identifying genes involved in common, genetically-complex epilepsies.

Voltage-Gated Sodium Channels

Voltage-gated sodium channels are responsible for the initiation and propagation of action potentials and are vital regulators of neuronal excitability. Voltage-gated sodium channel brain complexes were identified as a single α subunit associated with auxiliary β subunits[4]. The α structure contains four homologous domains (D1-D4), each consisting of six α–helical transmembrane segments (S1-S6)[5]. The S4 segments are positively charged and form the voltage sensor of the complex, which initiates channel activation[6-12] (Figure 1). Furthermore, there is evidence of S4/D4 having a unique function in inactivation[5,9,13]. The β subunits (β1-β4) are single transmembrane segments that modulate voltage dependence, kinetics and localization of the α subunits[14,15]. The α subunits primarily expressed in the brain are encoded by *SCN1A*, *SCN2A*, *SCN3A* and *SCN8A*[5,16-18]. *SCN1A* and *SCN3A* channels are located mainly in neuronal cell bodies. *SCN2A* channels are localized to dendrites, unmyelinated or pre-myelinated axons and *SCN8A* channels are found in dendrites and the nodes of Ranvier[19-23].

Voltage-Gated Sodium Channels and Epilepsy

Currently more than 700 mutations in *SCN1A* have been reported in patients with various types of epilepsy, making it the most common cause of monogenic epilepsy. Mutations in voltage-gated sodium channels are responsible for several types of human epilepsy, including Genetic Epilepsy with Febrile Seizures Plus (GEFS+) and Dravet Syndrome (DS), formerly known as Severe Myoclonic Epilepsy of Infancy[3]. GEFS+ is a benign, childhood-onset syndrome with autosomal dominant inheritance (OMIM 604233). This familial syndrome is characterized by febrile seizures that persist beyond six years of age and afebrile, generalized seizure types later in life[24,25]. In 1998, the GEFS+ mutation $SCN1B^{C121W}$ was identified in the β1 subunit gene[26]. The effect of this mutation results in impaired modulation of the sodium channel α subunit[27]. In 1999, linkage analysis of two large families identified a second GEFS+ locus localized to chromosome 2[28,29]. The nonrecombinant interval contains a sodium channel gene cluster that includes *SCN1A, SCN2A, SCN3A* and *SCN9A*. It was found that affected individuals from one family were heterozygous for $SCN1A^{R1648H}$ and affected members from another family were heterozygous for $SCN1A^{T875M}$ [30]. When $SCN1A^{T875M}$ and $SCN1A^{R1648H}$ mutations were heterologously expressed with β1 and β2 subunits, both GEFS+ mutations exhibited noninactivating inward sodium currents[31]. Additionally, single channel analysis of $SCN1A^{R1648H}$ activity revealed mutants had a higher probability of late channel openings[31]. The data suggest that these GEFS+ variants result in gain-of-function mutations caused by defects in sodium channel inactivation, thereby enhancing sodium current. Persistent sodium influx is hypothesized to extend neuronal depolarization, initiate hyper-excitability and increase seizure susceptibility[31].

DS is a disease most frequently associated with mutations of *SCN1A*[32]. Over 70% of reported *SCN1A* mutations have been identified in DS patients[32]. Approximately 90% of *SCN1A* mutations found in DS patients arose *de novo* and nearly half are nonsense, frameshift or splice site mutations that result in protein truncation[3,33,34]. This infant-onset syndrome is characterized by generalized clonic, generalized tonic-clonic or hemiclonic seizures. Although development is normal prior to disease onset, progression is often coupled to a severe decline of psychomotor and cognitive development[35,36]. DS patients develop other types of seizures, including absence and partial seizures and respond poorly to anti-epileptic drugs.

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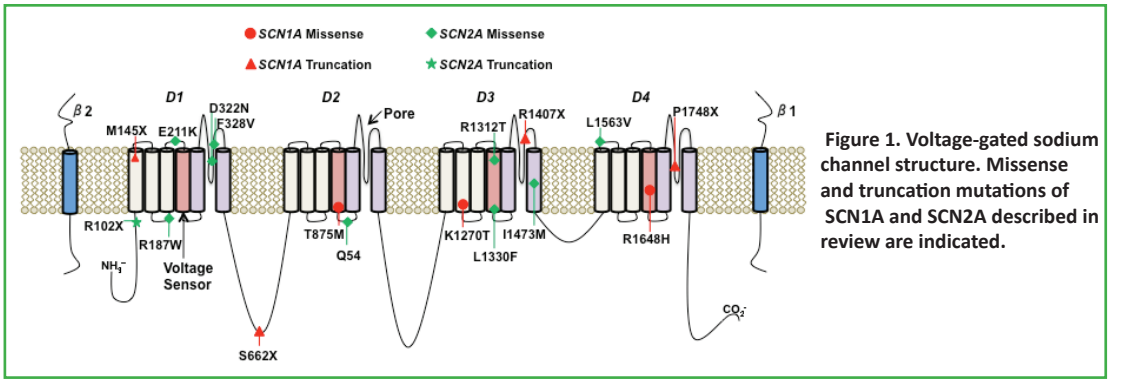

Figure 1. Voltage-gated sodium channel structure. Missense and truncation mutations of SCN1A and SCN2A described in review are indicated.

A smaller number of mutations have been reported in the other neuronal sodium channels *SCN2A, SCN3A* and *SCN9A. SCN2A* is a paralogous sodium channel gene closely linked to *SCN1A* on chromosome 2. Approximately 20 missense mutations in *SCN2A* have been detected in patients with GEFS+, Benign Familial Neonatal-Infantile Seizures (BFNIS) and DS[37]. The GEFS+ missense mutation *SCN2A*^{R187W} results in a delay of channel inactivation, hypothesized to cause persistent sodium current and repetitive firing during depolarization[38]. These abnormalities may be responsible for the hyperexcitability leading to seizures at the neuronal level[38]. BFNIS is characterized by the onset of afebrile generalized seizures typically between 2 days and 3.5 months of life, which spontaneously remit by age one[39]. *SCN2A*^{R1319Q}, *SCN2A*^{L1563V} and *SCN2A*^{L1330F} missense mutations were found in BFNIS patients[40,41]. Biophysical analysis revealed *SCN2A*^{R1319Q} and *SCN2A*^{L1330F} result in loss of channel function, with deficits in activation/inactivation and enhanced use-dependence, respectively[42]. *SCN2A*^{L1563V} impairs fast inactivation, resulting in a depolarizing shift in the voltage dependence[42]. Furthermore, all three mutations display significantly lower levels of protein expression at the cell surface[42]. These *SCN2A* mutants exhibit a wide range of functional abnormalities hypothesized to contribute to seizure generation. Missense mutations *SCN2A*^{D322N}, *SCN2A*^{F328V}, *SCN2A*^{R1312T}, *SCN2A*^{E211K} *and SCN2A*^{I1473M} and the nonsense mutation *SCN2A*^{R102X} were all identified in patients with DS[43-45]. *SCN2A*^{E211K} and *SCN2A*^{I1473M} mutations cause hyperpolarizing shifts in voltage dependence of activation which would be predicted to result in premature channel opening and hyperactivity[44]. The *SCN2A*^{R102X} mutation shifts the voltage dependence of inactivation in the hyperpolarizing direction, which is frequently associated with less channel availability, causing hypoexcitiablity[43]. Only one mutation of *SCN3A* has been identified in epilepsy. The missense mutation *SCN3A*^{K345Q} was found in an individual with partial epilepsy[46]. Functional analysis of *SCN3A*^{K345Q} revealed increased persistent current, decreased current threshold, spontaneous action potentials and paroxysmal depolarizing shift complexes[47]. These abnormalities are suggestive of epileptiform activities.

Variable Expressivity of Sodium Channel Mutations in Epilepsy

A common feature of inherited epilepsy due to sodium channel mutations is that family members who carry the same mutation often display a difference in the clinical severity of the disease. This is seen in both GEFS+ and DS. In a GEFS+ family carrying the *SCN1A*^{R1648H} mutation, four mutation carriers had epilepsy, one carrier had febrile seizures and seven had both[28,30]. Those with epilepsy had varied seizure types, including GTCS, myoclonic, partial, hemiclonic and absence seizures[28,30]. In another GEFS+ family with the *SCN1A*^{K1270T} mutation, 11 family members had only febrile seizures plus and five family members had evidence of temporal lobe epilepsy[48]. In rare cases, DS patients have inherited an *SCN1A* mutation from a mildly affected parent[49-51]. Gennaro and colleagues described a family in which two siblings with DS inherited the *SCN1A*^{P1748fsX1779} mutation from their mother, who had only a single febrile seizure in childhood[50]. Recently, Yu *et al*. identified two *SCN1A* truncation mutations that did not result in DS. Instead, *SCN1A*^{S662X} and *SCN1A*^{M145fsx148} produced GEFS+ and focal seizures, much milder forms of epilepsy not commonly associated with SCN1A truncations[52].

Variable expressivity suggests that other factors besides primary mutations influence the clinical manifestation of epilepsy. Contributing factors may include stochastic events during development, accumulation of somatic mutations throughout a lifetime or environmental insults such as trauma[53]. Variation in inheritance of genetic susceptibility alleles in different family members may also modify the clinical severity of epilepsy.

Epilepsy Models With Sodium Channel Mutations

Several mouse models with seizure-related phenotypes recapitulating GEFS+ and DS have been generated from mutations in voltage-gated sodium channel α subunits. A GEFS+ model was developed by knocking-in the *SCN1A*^{R1648H} mutation into the orthologous mouse gene[54]. To study DS, we have developed a targeted null allele of *Scn1a*, as has the Caterall laboratory[55]. Also, a knock-in of *Scn1a*^{R1407X} has been generated to examine the effects of truncated *Scn1a*[56].

SCN1A^{R1648H} was the first human *SCN1A* GEFS+ mutation studied *in vivo*. *Scn1a*$^{R1648H/R1648H}$ homozygous mice experienced premature lethality by postnatal day 26 (P26)[54]. Behavioral observations revealed both heterozygous and homozygous mutant animals exhibited spontaneous, generalized seizures that were confirmed by electrocorticography (ECoG) recordings[54]. Heterozygous mutants were more susceptible to seizure induction by the chemiconvulsant flurothyl and therefore had reduced times to seizure onset[54]. Flurothyl-induced seizure thresholds returned to wildtype levels after injection of the anticonvulsant valproic acid, commonly used to treat GEFS+ patients, validating the knock-in as a model for GEFS+[54]. Electrophysiological analysis demonstrated that cortical GABAergic bipolar interneurons from heterozygous and homozygous mice had reduced total sodium current amplitudes, increased use-dependence and slower recovery from inactivation[54]. Additionally, homozygous animals had a significant reduction in action potential firing in these interneurons[54]. It is hypothesized that reduced excitability of GABAergic interneurons is a key contributor to seizure generation in the *Scn1a*^{R1648H} GEFS+ model[54].

A DS mouse model was generated by disruption of the *Scn1a* gene. *Scn1a*$^{-/-}$ null mice exhibited spontaneous seizures and ataxia by P9, with premature lethality by P15[57]. *Scn1a*$^{+/-}$ heterozygous mice displayed frequent, spontaneous seizures that were confirmed by ECoG recordings[57]. *Scn1a*$^{+/-}$ heterozygotes experienced sporadic death between P21 and P27, with 40% lethality by the 15th week of life[57]. Electrophysiological analysis from hippocampal GABAergic interneurons revealed a significant reduction of sodium current levels in *Scn1a*$^{+/-}$ heterozygous and *Scn1a*$^{-/-}$ homozygous mice[57]. GABAergic interneurons also exhibited significant decreases in action potential firing, frequency and amplitude in both *Scn1a*$^{+/-}$ heterozygous

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and *Scn1a*$^{-/-}$ homozygous mice[57]. These abnormalities are indicative of reduced GABA transmission, a major contributor to neuronal hyperexcitability and seizure generation.

An additional knock-in model of DS was generated with a premature stop codon recapitulating the human *SCN1A*^{R1407X} mutation identified in three unrelated patients[49,56,58,59]. Mice homozygous for the mutation exhibited tonic-clonic seizures confirmed by ECoG starting at P12 and experienced premature lethality by P21[56]. Heterozygous *Scn1a*^{R1407X} mice developed seizures verified by ECoG at P21 with a 40% mortality rate by three months of age[56].

Our laboratory has developed a mouse model with a missense mutation of *Scn2a*, designated *Scn2a*^{Q54}, that resembles an epilepsy phenotype of human patients. The transgenic *Scn2a*^{Q54} mouse has a gain-of-function mutation [GAL(879-881)QQQ], located in the S4-S5 intracellular linker in D2. *Scn2a*^{Q54} mice have a progressive epilepsy phenotype which begins with brief, partial motor seizures[60]. As they age, *Scn2a*^{Q54} mice exhibit more frequent partial seizures, along with secondarily generalized seizures and have a reduced lifespan[60]. Recordings of isolated excitatory hippocampal pyramidal neurons from *Scn2a*^{Q54} mice revealed increased persistent sodium current which is hypothesized to contribute to seizure generation by increasing neuronal hyperexcitability[60]. Functional studies of several human GEFS+ mutations in heterologous expression systems have also shown increased persistent current of similar magnitude[31,61,62]. Hippocampal slice recordings from *Scn2a*^{Q54} mice demonstrated network hyperexcitability during recording of spontaneous and evoked activity, supporting the hypothesis that increased persistent current leads to neuronal hyperexcitability[63].

Genetic Background Influences Epilepsy Models

A common feature of mouse seizure models, including sodium channel mutants, is that seizure frequency and survival vary significantly depending on the genetic background of the mouse, indicative of genetic modifiers in epilepsy. Consequently, these models provide a useful system for identifying modifier genes that may also contribute to variable expressivity in human epilepsy patients. Genes that influence a mutant phenotype can be identified systematically by crossing the mutation onto different background strains. Modifier loci have been mapped for several diseases in human and mouse, including cystic fibrosis, cancer, retinal, cochlear and motor neuron degeneration, aganglionosis, otocephaly, tremor and dystonia[64-71]. Modifier genes have been identified for several of these loci[66,72-77].

Early studies revealed that there were considerable differences in seizure susceptibility dependent on genetic background[78,79]. Ferraro and colleagues demonstrated that genetic factors in C57BL/6J (B6) and DBA/2J (DBA) influence seizure susceptibility, with the B6 strain being far more seizure resistant than the DBA strain to a variety of seizure induction methods[80-85]. Genetic mapping approaches identified *Kcnj10*, an inward rectifying potassium channel, as a candidate gene for seizure susceptibility in these two strains[86-88].

Scn1a$^{+/-}$ heterozygotes on a B6 background have an 80% lethality rate by 13 weeks of age and frequent, spontaneous seizure activity[55]. When *Scn1a*$^{+/-}$ heterozygotes were bred to and maintained on a 129/SvJ background, only 10% of heterozygotes died by 15 weeks and seizure activity was not observed[55]. Loss of *Scn1a* is more severe on the B6 background, suggesting the 129/SvJ background contributes modifier genes that improve the epilepsy phenotype. Similarly, the *Scn1a*^{R1407X} mutation was found to be far less penetrant on the 129/SvJ background[56].

Scn2a^{Q54} mice on a B6 background exhibit a low incidence of seizures, with less than 20% having seizures at three months of age and more than 75% survive beyond six months of age[89]. When B6.*Scn2a*^{Q54} mice are crossed to the SJL/J (SJL) background, the resulting (SJL x B6)F1.*Scn2a*^{Q54} offspring display a high incidence of seizures (>80%) with early onset and only 25% survival to six months of age[89]. This observation indicates the SJL strain contributes dominant modifiers which affect the severity of the epilepsy phenotype[89]. Genetic mapping was performed which identified two modifier loci responsible for the strain difference in *Scn2a*^{Q54} mice: *Moe1* (Modifier of Epilepsy 1) and *Moe2*[89]. The voltage-gated potassium channel *Kcnv2* (Kv8.2) was found to be a strong candidate for *Moe2*[90].

Genetic Modifiers of Epilepsy

The contribution of genetic modifiers in influencing clinical severity is becoming increasingly important for understanding the pathophysiology of inherited disease. Several genes have already been identified in mouse models, which alter seizure phenotype.

Scn8a has been identified as a modifier of DS. The *Scn8a*$^{med\text{-}jo}$ mouse model contains a missense mutation that results in neuromuscular abnormalities, but has increased thresholds to seizure induction[91-96]. When *Scn8a*$^{med\text{-}jo/+}$ heterozygotes were crossed with *Scn1a*$^{+/-}$ heterozygotes to generate double heterozygous mutants, the *Scn8a*$^{med\text{-}jo}$ allele was able to rescue reduced seizure thresholds of *Scn1a*$^{+/-}$ mice and improve their premature lethality[96]. These findings suggest reduced *Scn8a* function can improve the *Scn1a* seizure phenotype.

The calcium channel mutant *Cacna1a*$^{tg/tg}$ recapitulates absence epilepsy and was identified as a modifier of the temporal lobe epilepsy model *Kcna1*. *Kcna1* mutants lack the Kv1.1 α-subunit of the Shaker-type potassium (K^+) channels and therefore the loss of K^+ channel regulation of action potential firing. *Kcna1*$^{-/-}$ null mutants experienced premature lethality with a 26% survival rate to ten weeks of age and frequent, severe, tonic-clonic seizures[97]. When *Cacna1a*$^{tg/tg}$ is crossed to *Kcna1*$^{-/-}$ nulls to generate double homozygous mutants, 87% of animals survived to ten weeks of age[97]. Additionally, double homozygous mutants lacked absence seizures and had a 60% and 80% reduction in tonic-clonic frequency and length of seizure, respectively[97]. The modifier effect is hypothesized to be the reduced neurotransmission of *Cacna1a*$^{tg/tg}$ diminishing *Kcna1*$^{-/-}$ hyperexcitability.

The voltage-gated potassium and sodium channel mutants *Kcnq2*^{V182M} and *Scn1a*^{R1648H} mutants were identified as modifiers of the *Scn2a*^{Q54} seizure model[97,98]. *Kcnq2*$^{V182M/+}$ heterozygous animals have a reduced threshold to seizure induction but no spontaneous seizure activity[99]. When *Kcnq2*$^{V182M/+}$ heterozygotes were combined with *Scn2a*^{Q54}, double heterozygous mutants experienced early-onset, tonic-clonic seizures and juvenile lethality by three weeks of age, unlike their single mutant littermates[100]. When *Scn1a*$^{R1648H/+}$ heterozygotes were combined with *Scn2a*^{Q54}, double heterozygous mutants experienced 100% mortality by P24 and frequent, spontaneous tonic-clonic seizures[98]. These modifier effects demonstrate that neuronal excitability is influenced by the net activity of multiple ion channels[98].

In support of human genetic modifiers in epilepsy, mutations have been identified in *CACNA1A*, *KCNJ10*, *KCNQ2* and *KCNV2* in patients with absence, generalized, BFNIS and partial epilepsies, respectively[101-106].

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Conclusion

Genetic epilepsies with complex inheritance likely account for approximately half of all epilepsies with unknown origin[107]. In complex inheritance, each individual gene may have only a small effect on clinical severity of epilepsy, but in combination with other risk genes, the additive effects can be devastating. Mutations in voltage-gated sodium channels are major contributors to genetic epilepsies. *SCN1A* mutations are found in ~70% of DS and ~10% of GEFS+ patients[108]. Among affected family members who possess the same mutation, clinical severity of the disease can be strikingly different. Increasing evidence suggests that other genes are modifying the primary mutation, resulting in a more or less severe phenotype.

Generation of mouse models recapitulating voltage-gated sodium and other neuronal ion channel mutations have allowed for more accurate models of DS, GEFS+, absence and temporal lobe epilepsies. These *in vivo* models are not only beneficial to assess pathophysiological consequences of ion channel mutations, but are also useful for studying the molecular mechanisms of mutations, thereby increasing our understanding of epilepsy.

An increasing amount of evidence has shown that seizure phenotypes from gene mutations are modified by the genetic strain background. Genetic approaches have identified several epilepsy modifier genes in mouse models, including *Cacna1a*, *Kcnj10*, *Kcnq2*, *Kcnv2*, and *Scn8a*. Patient mutations have also been identified in the human orthologs of these genes supporting a role for genetic modifiers in human epilepsy. Identification of modifier genes that improve or exacerbate epilepsy may increase the understanding of the molecular events involved in epileptogenesis, advance molecular diagnostic capabilities and identify novel therapeutic targets for improved treatment of human patients.

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CANDIDATE REVIEWS

Epilepsy associated mutations in the γ2 subunit of $GABA_A$ receptor

Xuan Huang

Abstract

Idiopathic generalized epilepsies are a group of neurological disorders with genetic origins. Genes coding for ion channels are involved in the disease development. Mutations identified from epilepsy patients have been located to type A γ-aminobutyric acid (GABA) receptor ($GABA_AR$). $GABA_ARs$ are GABA gated pentameric chloride channels mediating fast inhibitory neurotransmission in the brain. Nineteen homologous subunits have been cloned and γ2 containing receptors are the major native compositions. In this review, we discuss several well studied epilepsy associated mutations in γ2 subunit: the missense mutations *R43Q* and *K289M*, the premature translation termination codon (PTC)-generating mutations *Q351X* and *IVS6+2T→G*. Different etiology of these mutations will be addressed.

Keywords: *epilepsy, $GABA_AR$, missense mutation, PTC mutation*

Introduction

Epilepsy is a neurological condition affecting 1% of the population and is characterized by recurrent and usually unpredictable seizures, . A hyperexcitable or hypersynchronized state of the neuronal network underlie syndromes observed in epilepsy patients. Although acquired factors such as trauma and infection could cause epilepsy, idiopathic generalized epilepsies (IGE) are of familial origin[1-3]. Given neuronal excitability is controlled by various ion channels, impairments on channel activities could lead to disease development. Mutations in ion channels have been reported to associate with epilepsy, such as voltage gated sodium channel[4], voltage gated potassium channel[5], nicotinic acetylcholine receptor[6] as well as type A γ-aminobutyric acid (GABA) receptor ($GABA_AR$)[7-8].

GABA is the main inhibitory neurotransmitter in the central nervous system. One of the major postsynaptic targets activated by GABA is $GABA_AR$, through which chloride ions enter to hyperpolarize the cell membrane, maintaining inhibitory tone. Widely expressed across the brain, $GABA_ARs$ are targets of several pharmaceutical drugs such as benzodiazepine (BZD) and barbiturates; they are also modulated by neurosteroids[9-10]. Besides, $GABA_ARs$ are associated with multiple disorders, including depression[11], schizophrenia[12], alcoholism[13] and epilepsy. Pentylenetetrazol, a $GABA_AR$ antagonist, has been widely used to induce generalized clonic seizures in rodent model[14-15]. The oscillatory synchrony recorded in mice brain was dramatically intensified when the $GABA_AR$ mediated inhibition was abolished in the reticular nucleus[16]. Moreover, multiple mutations in $GABA_AR$ have also been revealed in genetic study on epilepsy patients[7-8,17], implying $GABA_ARs$ play a role in the pathophysiology of epilepsy.

Here we address how a variety of epilepsy associated mutations identified in γ2 subunit of $GABA_AR$ could impair the receptor function, and how these impairments might increase the epilepsy susceptibility. First, a discussion on the subunit composition of $GABA_AR$; next, several well studied mutations will be discussed.

$GABA_AR$: Subunit Composition

$GABA_ARs$ belong to the family of pentameric cys-loop ligand gated ion channels. In the mammals, this subfamily also includes nicotinic acetylcholine receptors (nAChR), serotonin type 3 receptors, and glycine receptors. Five homologous subunits are arranged pseudo-symmetrically to form a central ion passing pore. Similar to other subunits from cys-loop family, $GABA_AR$ subunits contain a large N terminal extracellular domain, followed by four transmembrane segments (M1~M4) and a small C terminal tail. The GABA and BZD binding sites are located in a different extracellular subunit interface, while the M2 segments line the inner pore[18-19].

Nineteen different $GABA_AR$ subunits, α1-6, β1-3, γ1-3, δ, θ, ε, π, and ρ1-3, have been cloned, and the heterogeneity is increased by alternative splicing[20], RNA editing[21], as well as posttranslational modification[22]. However, only certain subunit combinations could form functional receptors. Study on the unary, binary, and ternary combination of α1, β2 and γ2L subunits showed that although several distinct oligomers existed in endoplasmic reticulum (ER), only αβ and αβγ combinations could form functional channel on the surface. Immature oligomers were retained in ER and degraded quickly[23-24]. The major native receptor composition found in the brain is αβγ, but extrasynaptic αβ receptor was also reported[25]. Comparison of these two receptor types in mammalian cell lines revealed that αβ receptor displayed a lower single channel conductance, smaller macroscopic amplitude and higher zinc sensitivity[26-28] (Figure 1). However, the generation of αβ receptor seems to be relatively inefficient, as the αβγ composition is preferred in the presence of γ subunit. A 2α:2β:1γ receptor stoichiometry was suggested for αβγ composition[29-30], in a counterclockwise arrangement of γ-β-α-β-α when viewed from the extracellular space[31] (Figure 1 inset).

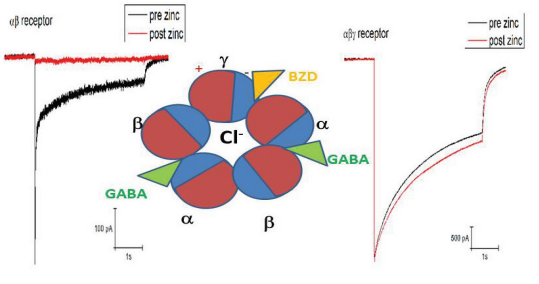

Figure 1: Whole cell recording showing GABA mediated chloride current through αβ and αβγ receptors. Black line: 1mM GABA only; red line: 1mM GABA + 10uM zinc2+ after 10s zinc2+ inhibition. The vertical scale bar is 100pA and 500pA respectively. The inset shows the schematic view of a pentameric αβγ receptor viewed from the synaptic cleft. Yellow triangle: BZD binding site; Green triangle: GABA binding site.

Epilepsy associated mutations in γ2 subunit

As a subunit of the major $GABA_AR$ isoform, γ2 subunit is distributed throughout the brain.

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Immunohistochemistry staining revealed strong signals in most regions including cerebral cortex, basal ganglia, hippocampus, and relatively weak signal in thalamus[32]. Postsynaptic clusters of $GABA_AR$ and gephyrin was greatly reduced when γ2 subunit was deleted[33]. The majority of homozygous γ2 knockout mice die within a few days after birth, and the surviving mice showed retarded growth, sensorimotor dysfunction and reduced life-span[34]; the heterozygous knock out displayed a higher level of anxiety[35]. Several epilepsy associated mutations have been identified in γ2 subunit (Figure 2): some are missense mutations where a specific residue is replaced by another; others generate a premature translation termination codon (PTC), stop codon caused by nonsense mutation, or frameshift mutation.

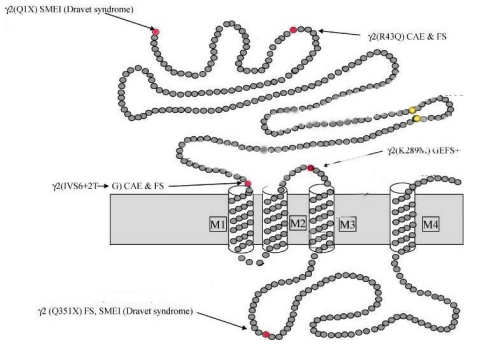

Figure 2: The topology of γ2 subunit of $GABA_AR$. The subunit is composed of a big extracellular N terminal region, four transmembrane domains (M1~M4) and a small C terminus. The red circles represent mutations identified in epilepsy patients. The yellow circles represent the two conserved cysteine residues.

Missense Mutations: *R43Q*

The *R43Q* mutation is one of the first epilepsy associated mutations identified in $GABA_AR$, and also the most extensively studied one. It was originally found in a large Australian family with epilepsy[8,36], segregating with patients with childhood absence epilepsy, febrile seizure or febrile seizure plus. A single nucleotide substitution was found in the *GABRG2* gene of these patients, causing a highly conserved arginine residue in γ2 peptide to be replaced by glutamine. This 43rd arginine in the mature peptide is located in the extracellular N terminus.

Several mechanisms have been suggested for *R43Q* mutation, including altered channel kinetics[37], interruption of BZD binding[8,37], decreased amplitude of GABA induced current[38-40], and decreased surface expression of γ2 subunit[39-43]. These discrepancies might be explained by the different heterologous expression systems and techniques. Initially, electrophysiological recordings in oocytes suggested this mutation disrupted BZD potentiation, decreasing the efficacy of inhibitory neurotransmission and generating a hyperexcitable state in the thalamocortical network[8]. This was supported by observations showing that arginine43 is inside one of the two important BZD binding domains[44]. Positron emission tomography revealed BZD binding was reduced in patients harboring *R43Q* mutation, especially in the anterior region of the brain[45]. However, as gamma subunit is necessary for BZD binding[33-34,46], a loss of γ2 subunit would also impair the BZD effects.

Biochemical evidences from surface biotinylation, radioactive ligand binding and immunofluorescence imaging agreed the *R43Q* mutation impaired the surface expression of γ2 subunit of $GABA_AR$, reducing the postsynaptic inhibitory response. According to the homology model derived from the crystal structure of acetylcholine binding protein, the R43 residue of the γ2 subunit was located in the face contributing to the β/γ interface rather than the α/γ interface where BZD binds. A salt bridge may connect the R43 and E178 residues in γ2 subunit with the R117 residue in β2 subunit. In addition, a pull down assay identified the 15-residue segment surrounding R43 capable to mediate subunit binding. This interaction was abolished when the arginine residue was changed to glutamine. Similarly, mutagenesis on neighboring Asp39 and Pro44 disrupted the membrane insertion of γ2 subunit. It is likely the conserved region around R43 plays a role in the receptor assembly and surface insertion of γ2 subunit[40-41,43], thus the mutant γ2 subunit was trapped in ER[39]. However, it is still controversial whether the retained mutant γ2 subunit would affect the surface insertion of its coupling partners. Overexpressing γ2(*R43Q*) subunit in hippocampal neurons, Eugene and colleagues found the mutant did not affect the synaptic IPSP, which is largely contributed by $GABA_ARs$ containing α1 subunit. However, the mutant reduced the extrasynaptic tonic currents by preventing the surface expression of α5 subunit[42]. Using an overexpressed amount of γ2 subunit in COS-7 cells, Frugier and colleagues suggested the retained γ2(*R43Q*) subunit did not affect the surface targeting of α3β3 complexes. A decreased number of α3β3γ2 receptors was accompanied with an increase of α3β3 receptors[43]. Flow cytometry data from our lab suggested that while a small amount of mutant αβγ receptor was expressed on the cell surface, a large amount of surface receptors switched to αβ receptor composition (unpublished data). As αβ receptors are extrasynaptic and exhibit smaller single channel conductance, this switch might affect the balance between tonic and phasic inhibition or decrease the efficiency of inhibitory neurotransmission.

A heterozygous knock-in mouse model carrying the *R43Q* mutation was generated[47]. The mutant mice displayed spontaneous absence seizures characterized by 5-8 Hz, high amplitude spike-and-wave discharges (SWDs) on electroencephalography, which could be blocked by antiepileptic drug ethosuximide. This is similar to the typical 3-4 Hz SWDs recorded in patients of childhood absence epilepsy[48]. The threshold of seizure induced by pentylenetetrazol was also significantly reduced in *R43Q* heterozygous mice. Interestingly, a small but significant reduced sIPSC was observed in cortical pyramidal neurons, but absent in thalamic reticular nucleus or ventralbasal thalamus neurons. How the mutant mice developed such specific deficit needs further investigation. Another conditional knock-in mouse model was also generated where the temporal expression of the γ2(*R43Q*) allele in the forebrain could be controlled[49]. When one copy of wild-type allele was normally expressed, activation of the *R43Q* allele increased the seizure threshold, and inactivation of the *R43Q* allele during development would decrease the threshold in adulthood. The epileptic phenotype of the heterozygous *R43Q* mice indicates a subtle reduction of cortical inhibition might underlie the pathogenesis of epilepsy. The expression of one *R43Q* allele seems to increase the seizure susceptibility compared to haploinsufficiency, and the changed channel activity during development might play an important role in disease onset in later life.

Generally, the *R43Q* mutation disrupts surface expression of the γ2 subunit. This could be caused by deficit of the subunit oligomerization or membrane insertion. The *R43Q* mice recapitulate some epilepsy syndromes, indicating disruption of $GABA_AR$ could result in the hyperexcitable state observed in epilepsy.

K289M

K289M mutation was found to segregate with various phenotypes of generalized epilepsy with febrile seizure plus in a French family[7]. It was

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caused by a single nucleotide change, converting a highly conserved lysine residue in the M2-M3 linker of γ2 subunit to methionine.
This mutation might cause decreased amplitude[7,50] or altered kinetics[38,42] of GABA evoked macroscopic current. The lysine residue in the extracellular M2-M3 linker is conserved among $GABA_AR$ and glycine receptor subunits, participating in channel gating[51]. During gating, the homologous K278 residue in the M2-M3 linker of α1 subunit was proposed to interact electrostatically with negative charged aspartic acid residues in the subunit interface. In the presence of GABA, K278 of the α1 subunit was in proximity to extracellular D149 in loop 7, indicating these two regions move closer during gating process[52]. Single channel recordings showed the mean open time of γ2(K289M) containing receptors was briefer, similar to α1(K278M) containing receptors[38,53]. The channel opening equilibrium constant of γ2(K289M) was also shown to be five fold lower than wild-type[50]. Although reduced macroscopic current amplitude was identified in oocytes and mammalian cells[7,50], no such difference was observed by some other groups[38,42,53]. Instead, both macroscopic currents elicited by rapid agonist application on HEK cells and mIPSCs of hippocampal neurons showed accelerated deactivation rate of γ2(K289M) mutation. The faster deactivation of $GABA_AR$ could also result in reduced inhibitory neurotransmission, as the total volume of chloride ion transferred is reduced. No significant change of protein expression or channel conductance was identified[42,53]. Compared to the R43Q mutation destroying surface targeting, K289M mutation affects the channel activity of type A GABA receptors.

PTC mutations: *Q351X*

Q351X is a nonsense mutation identified in a family with GEFS+[54], and the proband was diagnosed with the severe myoclonic epilepsy in infancy. A single nucleotide substitution in exon 9 of *GABRG2* was identified in three members of this family, suggested to form part of the epilepsy inheritance pattern. This mutation results in a premature stop codon in the glutamine351 residue of mature γ2 subunit.

The *Q351X* mutation displays dominant negative effects. As glutamine351 is located in the big M3-M4 cytoplasmic loop, a truncated peptide lacking the fourth transmembrane domain and the small C-terminal tail was generated by *Q351X* mutation. Expression of mutant γ2(*Q351X*) with α1 and β2 subunit in oocytes and HEK cells showed the GABA induced currents were completely abolished and mutant γ2 subunit was retained in ER complex. The detrimental consequence of this mutation suggested the mutant γ2 subunit might also affect the assembly of α1 and β2 subunits into functional receptor[54]. Kang *et al*. studied the protein expression and channel function of γ2(*Q351X*) in different conditions. They compared the hemizygous condition where half dosage of the wild-type subunit is transfected,with heterozygous condition where half dosage of the wild-type and half dosage of the mutant subunit are co-transfected.They found that the mutant γ2 subunit was trapped in the ER, and also prevent the membrane insertion of wild-type α1, β2 and even γ2 subunits through subunit oligomerization[55].

Premature truncation could activate surveillance mechanisms including nonsense-mediated decay (NMD) and ER associated degradation (ERAD)[56]. NMD is a posttranscriptional process to eliminate mRNA that would cause prematurely terminated translation; generally PTCs located more than 50-55 nt upstream of an exon-exon junction could trigger NMD[57]. ERAD is an ER quality control mechanism to eliminate misfolded peptides[58]. Utilizing minigene containing intron 8 sequence, it was shown the mRNA level of the mutant γ2(*Q351X*) was not affected, accordant with the fact the glutamine351 in the last exon is unable to trigger NMD. However, on the protein level, the α1 subunit was degraded more rapidly in the presence of γ2(*Q351X*) subunit, through the proteosome mediated ERAD[55].

To conclude, *Q351X* mutation generated a truncated γ2 subunit with trafficking deficit. The mutant γ2 subunit would retain oligomerization partners in ER and accelerate their degradation. The mutant γ2 subunit also affects the membrane insertion of wild type γ2 subunit, exhibiting a dominant negative effect.

IVS6+2T→G.

γ2(IVS6+2T→G) is an intronic mutation segregated with childhood absence epilepsy and febrile seizures in a small pedigree. It is a single nucleotide change identified in the splice donor site of the intron 6 of *GABRG2*. This mutation could cause exon skipping or alternative splicing using cryptic splice donor sites. Premature truncated protein was predicted for both situation, due to the presence of in-frame stop codons[59].

In contrast to the *Q351X* mutation, the amount of mutant *γ2(IVS6+2T→G)* was affected by NMD. Splicing is a posttranscriptional event to remove introns, which greatly enhances protein diversity. During a typical splicing process, the spliceosome recognizes the 5' terminal splice donor site with dinucleotide GT sequence and the 3' terminal splice acceptor site with AG sequence, followed by intron excision[60]. Intron splicing is affected by some intronic RNA motifs, such as sequences around the donor sites and acceptor sites. Mutations identified in these motifs have been associated with diseases including cancer[61]. Mutations in the splice donor site could cause exon skipping, intron retention or use of cryptic splice sites[62]. Tian *et al*. investigated on the effects of the *γ2(IVS6+2T→G)* mutation using minigene and bacterial artificial chromosome (BAC). A cryptic splicing donor site was activated by the mutation: part of the intron 6 sequence was retained, causing a frame shift in exon 7 and producing a premature stop codon. NMD was shown to be activated by this PTC, and the amount of transcript was reduced[63].

Not all mutant transcripts are cleared by NMD in *IVS6+2T→G* mutants, as a truncated γ2 peptide without transmembrane domain was still produced. Expressed in HEK cells, the mutant γ2 peptide was retained in ER and could not oligomerize with other subunits[63]. In a word, *γ2(IVS6+2T→G)* mutation results in a nonfunctional protein. Different from the dominant negative effects of *Q351X* mutation, the pathogenesis of patients carrying this mutation might be the consequence of haploinsufficiency of γ2 subunit.

Conclusion

As one of the main ion channels mediating inhibitory signal, $GABA_AR$ is involved in the pathogenesis of epilepsy. Here we discuss four well studied mutations identified from small pedigrees, showing impairments on γ2 subunit of $GABA_AR$ could increase the epilepsy susceptibility. In future studies, it will be important to investigate 1) whether these mutations are the main genetic factor in these pedigrees; 2) whether distinct epilepsy subtypes are contributed by different mechanisms of channel dysfunction; 3) how different mutations or polymorphisms work together to affect the individual seizure threshold. A combination of traditional electrophysiology and biochemistry methods and transgenic techniques would improve our understanding of this area.

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CANDIDATE REVIEWS

The Regulation of Cell Survival by the p75 Neurotrophin Receptor

Bradley R. Kraemer

Abstract

During the development of the mammalian nervous system, the p75 neurotrophin receptor ($p75^{NTR}$) can fulfill dual functions. Through the activation of the stress-activated kinase JNK or by stimulation of $p75^{NTR}$-associated factors, the receptor can induce pro-apoptotic pathways. In other contexts, $p75^{NTR}$ can promote cell survival by associating with tropomyosin-related-kinase (Trk) receptors or by independently promoting activation of the transcription factor NFκB. Though these signaling processes are not fully understood, recent studies have indicated that the regulated proteolytic cleavage of $p75^{NTR}$ may play a particularly important role. Moreover, analyses of $p75^{NTR}$-associated receptors and cytoplasmic interactors have provided new $p75^{NTR}$ into the mechanisms by which $p75^{NTR}$ mediates these functions. This review discusses the signaling events associated with the regulation of cell survival by $p75^{NTR}$, highlighting how these activities may contribute not only to neurodevelopment, but also to cellular responses to stressful or injurious conditions.

Keywords: *p75ntr, neurotrophins, NGF, BDNF, TRAF6, NRIF, NRAGE, apoptosis, sortilin*

Introduction

The neurotrophin family, which consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4), is a group of secreted proteins which mediate a wide range of biological functions, including the regulation of cell survival, myelin formation, neurite outgrowth, synaptic plasticity, neuronal differentiation, and cell migration[1,2]. Neurotrophins perform these many functions by acting through two classes of receptors: the Trk family of receptor tyrosine kinases (TrkA, TrkB, and TrkC), and the p75 neurotrophin receptor ($p75^{NTR}$), a member of the Tumor Necrosis Factor (TNF) superfamily[1,2]. Trk receptors preferentially bind to particular neurotrophins, with TrkA selectively associating with NGF, TrkB with BDNF and NT-4, and TrkC with NT-3[3,4]. Depending upon the cellular context, these interactions can activate well-studied phosphatidylinositol-3-kinase (PI-3 kinase)/Akt, Ras/extracellular signal–regulated kinase (ERK), and phospholipase C-γ (PLC-γ) signaling pathways, as well as other signaling cascades, and thereby regulate a diversity of physiological activities[4]. In contrast to Trk receptors, $p75^{NTR}$ has a similar affinity for all four neurotrophins, and the signaling events associated with its activation are less well characterized[2,3]. The established functional roles of $p75^{NTR}$ are quite diverse, due largely to abundance of ligands and co-receptors that can associate with $p75^{NTR}$ and regulate its signaling. For example, $p75^{NTR}$ can form a high-affinity complex with Trk receptors and thereby enhance Trk signaling[1,5]. By associating with the Nogo receptor and Lingo-1 in the presence of myelin-based ligands, $p75^{NTR}$ can also inhibit the axonal regeneration of injured neurons[1,6,7]. Numerous other functional roles for $p75^{NTR}$ have been established, including modulation of the cell cycle[8], synaptic plasticity[9], and tumor cell migration[10]. This review discusses the mechanisms by which $p75^{NTR}$ regulates cell survival and how these events relate to neurodevelopment and to physiological responses to cellular injury.

P75NTR Activates Programmed Cell Death

Programmed cell death is an essential aspect of mammalian neurodevelopment, as approximately 50% of neurons generated during development undergo an apoptotic program[11]. Analyses from *p75ntr*$^{-/-}$ mice have revealed that $p75^{NTR}$ is vital to this process and induces the developmental loss of neurons within the retina[12,13], basal forebrain[14], spinal cord[13], and superior cervical ganglia[15]. Additionally, *in vitro* studies have shown that neurotrophin binding, or neurotrophin withdrawal, induces $p75^{NTR}$-mediated apoptosis in a plethora of cell types, including Schwann cells[16,17], oligodendrocytes[18], hippocampal neurons[19], motor neurons[20], photoreceptor cells[21], sympathetic neurons[22], and several others[23-25]. The signaling cascades induced by $p75^{NTR}$-mediated apoptosis are not completely understood, but are known to involve the phosphorylation of c-Jun N-terminal kinase (JNK)[26-28], as well as the downstream activation of p53[29] and BH3-domain-only family members Bad[27] and BimEL[26], the accumulation of cytochrome c within the cytosol[27], and the activation caspases-3, -6, and -9[27,28]. This is in contrast to other members of the TNF superfamily, which activate apoptosis through a caspase-8-dependent pathway[30]. Exactly how activation of $p75^{NTR}$ leads to these downstream activities is incompletely understood, but many studies have recently identified critical early signaling events. One such important early event is proteolytic processing of the receptor. In a manner similar to that of the transmembrane proteins Notch and amyloid precursor protein (APP), $p75^{NTR}$ is subject to proteolytic cleavage in its extracellular domain (ECD) by the metalloproteinase[A] TNF-α converting enzyme (TACE, also known as ADAM17), thereby producing a membrane-bound c-terminal fragment (CTF). Following release of the ECD, the $p75^{NTR}$-CTF is cleaved in its transmembrane region by γ-secretase, thus releasing into the cytoplasm the intracellular domain (ICD) of $p75^{NTR}$ [2,31,32]. This type of proteolytic processing of $p75^{NTR}$ has been demonstrated to occur in a ligand-dependent manner in 3T3-$p75^{NTR}$ over-expressing cells[33], glial cells[34], and sympathetic neurons[2,35]. Cleavage of $p75^{NTR}$ in response to phorbol ester[B] treatment has been revealed in HEK-293 cells expressing $p75^{NTR}$ [32], SN56 cells[32], and RNF22 Schwannoma cells[31]. In other contexts, $p75^{NTR}$ cleavage has been demonstrated to occur in PC12 cells[36], dorsal root ganglion (DRG) neurons[37], cerebellar granular neurons (CGNs)[38] and in a transgenic photoreceptor cell line[39]. The functional significance of these proteolytic events is, in many aspects, not well understood and likely varies depending upon the cellular context. One possibility is that γ-secretase-mediated cleavage of the $p75^{NTR}$-CTF allows for the translocation of the $p75^{NTR}$-ICD into the nucleus, where it may alter transcriptional events that contribute to apoptotic signaling. Indeed, the soluble $p75^{NTR}$-ICD has been detected in the

[A] **Metalloproteinases**: a group of peptidases which require metals such as zinc or calcium for catalytic function.
[B] **Phorbol esters**: poly-cyclic compounds commonly used as diacylglycerol analogues which bind and activate protein kinase C.

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nucleus of both 3T3-cells over-expressing p75^{NTR} [33] and Schwann cells[34] in a ligand-dependent manner. In PC12 cells, NGF stimulation induced nuclear localization of endogenous p75^{NTR}-ICD and association of p75^{NTR} with the cyclin E promoter[40]. Altogether, this evidence suggests that proteolytic cleavage of p75^{NTR} may promote regulation of transcriptional activity by facilitating nuclear entry of the p75^{NTR}-ICD.

Another function of p75^{NTR} cleavage is to allow proteins associated with the p75^{NTR}-ICD to be released into the cytoplasm, thus allowing these interactors to undergo nuclear translocation or to associate with other cytoplasmic proteins. Numerous p75^{NTR} interacting factors have been reported, including NRIF[41], NADE[42], NRAGE[43], RIP-2[17], Rac[44], Rho-GDI[45], SC-1[46], MAGE-H1[47], Necdin[47], ARMS[48], and TRAF2, 4, and 6[49]. Many of these have been shown to mediate apoptotic signaling, including NRIF[41], TRAF6[50], NRAGE[51], and NADE[42]. Among these p75^{NTR}-associated proteins, the E3 ubiquitin ligase TRAF6 (TNF receptor-associated factor 6) plays a particularly important role. TRAF6 is recruited to the cytoplasmic domain of p75^{NTR} in a ligand-dependent fashion and is required for p75^{NTR}-mediated activation of JNK and subsequent neuronal death[50,52-54]. Recently, it was reported that following treatment of PC12 cells with NGF, TRAF6 associates with presenilin-1, the primary catalytic component of the γ-secretase complex, and this association enhanced ubiquitylation of p75^{NTR}, cleavage of p75^{NTR}, and autoubiquitylation of TRAF6[54]. While the functional significance of p75^{NTR} ubiquitylation is still unclear, this evidence suggests that TRAF6 may enhance p75^{NTR} cleavage by binding both p75^{NTR} and γ-secretase in response to stimulation by neurotrophins. The widely expressed DNA-binding protein NRIF (neurotrophin receptor-interacting factor) is another cytoplasmic interactor that facilitates p75^{NTR}-mediated apoptosis[35]. NRIF is required for p75^{NTR}-mediated JNK activation and apoptosis in cultured sympathetic neurons[55], and NRIF was recently shown to be necessary for p75^{NTR}-mediated apoptosis in hippocampal neurons both *in vivo* and *in vitro*[19]. In sympathetic neurons, pro-apoptotic ligands stimulate proteolytic cleavage of p75^{NTR}, which in turn induces the ubiquitylation and nuclear translocation of NRIF[35]. Inhibition of p75^{NTR} proteolysis by a γ-secretase inhibitor or by expression of a mutant, non-cleavable form of p75^{NTR} blocked nuclear translocation of NRIF and prevented neuronal death. Ubiquitylation of NRIF, which is required for its nuclear localization, as well as for p75^{NTR}-mediated apoptosis, also depends upon TRAF6[56]. Thus, in a current model of p75^{NTR}-mediated apoptosis, neurotrophin-dependent activation of p75^{NTR} induces cleavage of the receptor by TACE and γ-secretase, with a potential enhancement of this cleavage occurring through an interaction between presenilin-1 and TRAF6. Cleavage of p75^{NTR} then results in the release of a complex containing the p75^{NTR}-ICD, NRIF, and TRAF6 into the cytoplasm. In turn, this facilitates the TRAF6-dependent ubiquitylation and nuclear localization of NRIF, as well as the activation of JNK, thereby triggering downstream signaling events that induce neuronal death (Fig. 1).

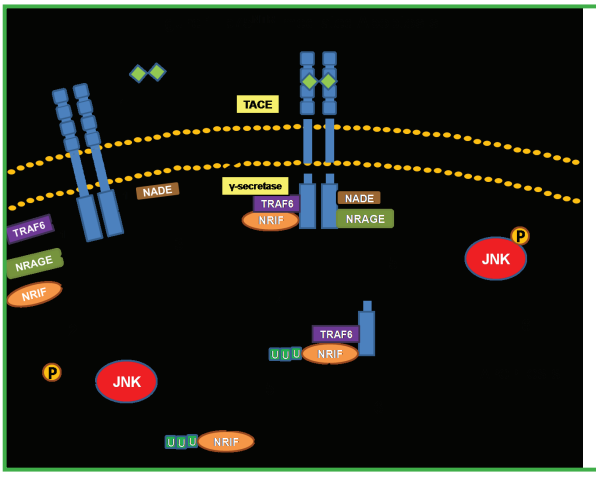

Figure 1: Activation of p75NTR by mature neurotrophins(NTs) or proNTs induces recruitment of p75NTR –associated factors and short-term JNK activation. JNK promotes cleavage of p75NTR by TACE and γ-secretase, thereby facilitating the release of a complex containing the p75NTR –ICD, TRAF6, and NRIF. This complex promotes the TRAF6-dependent ubiquitylation and nuclear translocation of NRIF, as well as the long-term activation of JNK, both of which lead to apoptosis. NRAGE, NADE, and other interactors may also contribute to this process, though the details of their interactions are currently unknown.

While this represents a plausible model of p75^{NTR}-mediated apoptosis, the roles of NRIF and TRAF6 in cell types other than sympathetic and hippocampal neurons are less well understood, and thus further investigation of potential cell-specific differences is needed. Additionally, other p75^{NTR}-associated factors are known to contribute to apoptotic signaling. NRAGE (neurotrophin receptor-interacting MAGE homolog), a member of the MAGE family, is a p75^{NTR}-associated protein that potently induces p75^{NTR}-dependent cell death when over-expressed in MAH cells treated with neurotrophins[43]. Recently, it was discovered that NRAGE knockout mice have a defect in the developmental apoptosis of sympathetic neurons similar to that observed in p75^{NTR} knockout mice, suggesting that NRAGE may be required for p75^{NTR}-mediated developmental apoptosis *in vivo*. Sympathetic neurons from these mice were resistant to BDNF-induced apoptosis and showed reduced BDNF-dependent JNK activation[51]. Thus, NRAGE appears to be an important component of p75^{NTR}-mediated apoptotic signaling. Interestingly, NRAGE knockout animals have a severe motor neuron defect not seen in p75^{NTR} knockout mice, indicating that NRAGE may also regulate apoptosis through pathways not involving p75^{NTR} [51]. NADE (p75^{NTR}-associated cell death executor) is another p75^{NTR}-associated protein that is thought to contribute to apoptotic signaling. When co-expressed in HEK293 cells, NADE associates with p75^{NTR} in response to NGF treatment and activates p75^{NTR}-dependent cell death, and these results were replicated in PC12 cells, nnr5 cells, and oligodendrocytes[42]. Whether NADE and NRAGE function within the same pathway as NRIF and TRAF6 or instead act in parallel to promote p75^{NTR}-mediated apoptosis is not well understood, and thus further analyses of these signaling events in similar cell types is needed.

p75^{NTR}-mediated Apoptosis in Response to Cellular Injury – Role of Proneurotrophins

In addition to regulating developmental apoptosis, it is well established that p75^{NTR} induces programmed cell death in response to different types of cellular injury. For example, p75^{NTR} facilitates cell death in corticospinal neurons following axotomy[57], in hippocampal neurons in response to seizures[19], in oligodendrocytes after spinal cord injury[58], and in motor neurons following lesion of the facial nerve[59,60]. Recently, a study conducted using cultured sympathetic neurons revealed that the neurotrophin-dependent induction of p75^{NTR} cleavage requires the activity of JNK. Interestingly, activation of JNK by over-expression of the upstream kinase MEK kinase-1 (MEKK1) was sufficient to induce p75^{NTR} cleavage and subsequent cell death[61]. Many types of cellular insults, including oxidative stress and ultraviolet irradiation, can activate JNK[62], and thus the activation of JNK by these different types of cellular stressors may stimulate p75^{NTR} cleavage and initiate subsequent apoptotic signaling. The precursor forms of neurotrophins also play an especially important role in stimulating p75^{NTR}-mediated cell death in response to neuronal injury. Like many secreted proteins, neurotrophins are synthesized as precursors (proneurotrophins), which can be proteolytically cleaved to produce mature proteins[9]. Proneurotrophins can be cleaved intracellularly by furin and other proconvertases or secreted in their precursor form and proteolytically cleaved by enzymes within the extracellular matrix[63,64]. The serine protease plasmin, which is activated through the proteolysis of plasminogen by tissue plasminogen activator (tPA),

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is one such enzyme capable of cleaving proneurotrophins within the extracellular environment[30,64]. Additionally, proneurotrophins can be converted by extracellular matrix metalloproteinases (MMPs), and, in particular, it has been demonstrated that proBDNF can be cleaved by MMP-3 and MMP-7, while proNGF is cleaved by MMP-7, but not MMP-2, -3, or -9[30,64]. Accumulating evidence has revealed that secreted proneurotrophins serve as potent endogenous ligands for $p75^{NTR}$. Proneurotrophins can activate $p75^{NTR}$ at low nanomolar concentrations by binding to a protein complex containing $p75^{NTR}$ and its co-receptor sortilin, a member of the Vps10p-domain family of receptors[65]. Proneurotrophins are thought to bind to sortilin via their pro-domain and to $p75^{NTR}$ through their mature domain, thus serving as a crosslinker that brings the three proteins together and initiates $p75^{NTR}$-mediated apoptotic signaling[65]. The death-promoting activity of proneurotrophins has been demonstrated in a variety of cell types, including oligodendrocytes[58], basal forebrain neurons[23], sympathetic neurons[22,65], Schwann cells[65], photoreceptor cells[21], corticospinal neurons[57], smooth muscle cells[64], and hippocampal neurons[19]. In several contexts, proneurotrophins have been demonstrated to promote programmed cell death in response to cellular injury *in vivo*. Exogenous proBDNF was shown to enhance the apoptosis of axotomized sensory neurons *in vivo*, and neutralizing antibodies which specifically bind the pro-domain of proBDNF reduced cell death following sciatic nerve lesion[66]. In hippocampal neurons, proNGF and proBDNF were upregulated after pilocarpine-induced seizures, particularly within astrocytes, and secretion of these proneurotrophins was detected in cerebrospinal fluid. Infusion of neutralizing proNGF antibodies into the hippocampal region reduced seizure-induced neuronal loss[19]. Following internal capsule lesion, an increase in the production and secretion of proNGF was detected, and infusion of neutralizing proNGF antibodies rescued corticospinal neurons from axotomy-induced cell death[57]. Because proneurotrophins have a greater affinity for $p75^{NTR}$/sortilin complexes and lower affinity for Trk receptors[65], proneurotrophins can selectively stimulate apoptosis in cellular systems in which mature neurotrophins promote Trk-mediated survival. Indeed, in cultured basal forebrain neurons, proNGF and proBDNF both induced $p75^{NTR}$-mediated programmed cell death, while the mature forms of these proteins instead activated pro-survival signaling pathways, presumably by stimulating TrkA and TrkB[23]. Apart from the differences in affinity for Trk receptors and $p75^{NTR}$, how the $p75^{NTR}$-mediated apoptotic signaling pathways stimulated by proneurotrophins differ from those induced by mature neurotrophins remains to be fully established. At least in some cellular contexts, the signaling mechanisms appear to be similar. For example, in sympathetic neurons, the induction of $p75^{NTR}$ cleavage and NRIF nuclear translocation by mature BDNF could also be invoked by lower concentrations of proBDNF[35,56]. Proteolysis of $p75^{NTR}$ and nuclear localization of NRIF were also required for the cell death of hippocampal neurons treated with proneurotrophins in culture[19]. Therefore, these signaling events appear facilitate $p75^{NTR}$-mediated cell death induced by both proneurotrophins and mature neurotrophins. However, sortilin may also contribute to $p75^{NTR}$-mediated apoptotic signaling in a manner that has yet to be revealed, and the involvement of other $p75^{NTR}$ interactors has not been thoroughly studied.

$p75^{NTR}$ Regulates Pro-survival Signaling

In contrast to the aforementioned role of $p75^{NTR}$, in some cellular contexts $p75^{NTR}$ can promote cell survival. Neurotrophin-dependent activation of $p75^{NTR}$ has been demonstrated to inhibit the death of neuroblastoma cells[67], of hippocampal neurons treated with NMDA[68], and of both sensory neurons[69] and cortical subplate neurons deprived of trophic support[70]. Mice lacking expression of $p75^{NTR}$ exhibit a dramatic loss of sensory neurons, thus indicating that the receptor is required for the survival of these neurons *in vivo*[71]. Additionally, a recent study of $p75^{NTR}$ knockout mice revealed an increased loss of primary auditory neurons following acoustic trauma, demonstrating that $p75^{NTR}$ may play a protective role following certain types of cellular injury[72]. As one mechanism of promoting cell survival, $p75^{NTR}$ can form a high affinity complex with Trk receptors, thereby increasing their specificity for particular neurotrophins[5,73]. Alternatively, stimulation of $p75^{NTR}$ can promote the activation of nuclear factor kappaB (NFκB), a pro-survival transcription factor that is also activated by other members of the TNF receptor family[60]. Neurotrophin binding to $p75^{NTR}$ has been shown to activate pro-survival signaling by NFκB in a number of cell types, including RN22 cells[74], primary Schwann cells[17], trigeminal neurons[75], and hippocampal neurons[76]. Activation of NFκB can, in turn, promote the expression of neuroprotective NFκB target genes such as Bcl-2 and Bcl-xl[76]. Recently, it was demonstrated that proteolytic cleavage of $p75^{NTR}$ might contribute to pro-survival signaling as well. Interestingly, activation of Trk receptors in PC12 cells, as well as in cerebellar granular neurons, was shown to induce cleavage of $p75^{NTR}$, which subsequently enhanced Trk signaling[36,38]. This function of $p75^{NTR}$ cleavage, however, is likely specific to particular cell types, since cleavage of $p75^{NTR}$ is not stimulated by Trk receptor activation in sympathetic neurons and expression of the $p75^{NTR}$-ICD in these cells promotes apoptosis[35].

Conclusion

Substantial progress has been made in uncovering the mechanisms by which $p75^{NTR}$ regulates cell survival during neurodevelopment and in response to cellular injury. Elucidating these processes is challenging due to cell-specific differences in $p75^{NTR}$ signaling and due to the complexity of the interactions of the receptor with multiple functionally specific signaling pathways. Despite these challenges, within many cellular contexts, the mechanisms of $p75^{NTR}$ signaling are becoming better understood. The association of $p75^{NTR}$ with sortilin or Trk receptors, the proteolytic cleavage of $p75^{NTR}$, and the stimulation of $p75^{NTR}$-associated factors represent key events in the regulation of cell survival by the receptor. Further analyses of $p75^{NTR}$ interacting factors and their activities within different cell types are needed, and the relationship of these activities to signaling events modulated by Trk receptors remains to be fully elucidated. Investigations along these lines will significantly enhance our understanding of the molecular processes through which $p75^{NTR}$ regulates cell survival.

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Corticotropin-Releasing Hormone in the Central Nucleus of the Amygdala: Link to Psychiatric Disorders

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Abstract
Corticotropin-releasing hormone (CRH) is a modulatory peptide that plays an essential role in the activation of the stress response. In the event of chronic stress, dysregulation of CRH expression occurs and is implicated in the pathology of psychiatric disorders. Individuals with depression and post-traumatic stress disorder (PTSD) show elevated levels of CRH protein in their cerebrospinal fluid. The source of this pathological increase in CRH expression is believed to be extrahypothalamic. One suspected extrahypothalamic source of CRH is the central nucleus of the amygdala (CeA). Normally, CRH in the CeA is believed to mediate autonomic and behavioral aspects of the stress response. However, dysregulation of this system has been linked to increased susceptibility to psychiatric disorders. The mechanism by which this increase in CRH levels in the CeA raises the risk for psychiatric disorders has not yet been elucidated. This paper will review current studies regarding the role of CeA CRH release in mediating the stress responses and how this relates to psychiatric disorders.

Keywords: *Corticotropin-releasing Hormone (CRH), Central nucleus of the amygdala (CeA), Lentivirus, Tetracycline-inducible System, Hypothalamic-pituitary-adrenal (HPA) axis*

Introduction

The Stress Response

Stress is a physiological response to a disruption in homeostasis caused by a physical or psychological element called a stressor[1]. The brain responds to stress by activating the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. The former system functions to rapidly increase the release of epinephrine in order to mediate autonomic functions[2,3]. This results in increased arousal, vigilance and a decision to 'fight or flight'[2,3]. On the other hand, the HPA axis mediates the slow and sustained aspects of the stress response and is linked to corticotropin-releasing hormone (CRH)[A] mRNA increase in the amygdala[4].

The HPA axis is a system of interactions between the hypothalamus, pituitary gland and adrenal gland that regulate hormonal responses to internal or external stimuli. Stress activates the HPA axis, causing the secretion of CRH and vasopressin (AVP) from the paraventricular nucleus (PVN) of the hypothalamus to activate corticotroph cells in the anterior pituitary[1]. There, both hormones induce synthesis of adrenocorticotropin releasing-hormone (ACTH), which causes corticosterone (CORT) secretion from the adrenal cortex into the bloodstream[1] (Figure 1). CORT then binds to glucocorticoid receptors (GR), expressed in peripheral organs and limbic brain regions such as the amygdala, thereby facilitating responses aimed at adapting to the stressor[1]. Moreover, CORT binding to GR in the central nucleus of the amygdala (CeA)[5] has been demonstrated to increase CRH mRNA synthesis. This has been demonstrated in studies where CORT pellet implantation[4,6] or repeated CORT injections[7] elevated CRH mRNA in the CeA of rodent models[4,6-8]. Consequently, when GR is deleted in the CeA, it results in a subsequent decrease in the levels of CRH mRNA[9]. As a mechanism of control, after GR is occupied by CORT, the sustained increase in levels of plasma CORT cause it to negatively feedback at the level of the PVN and anterior pituitary to inhibit its own secretion, and thus return the system to homeostatic levels[1]. However, in the event of chronic stress, this hormonal response of the HPA axis is hyperactivated causing an aberrant rise in CORT that is resistant to negative feedback to inhibit its secretion[10,11]. Additionally, further increases in CRH mRNA in the amygdala leads to changes that heighten maladaptive emotional behavior[12]. This rise in amygdala CRH activates the HPA axis[13], possibly though CeA interaction with its targets described later in this review.

The CEA in Psychiatric Disorders

The aforementioned dysregulation of the HPA axis plays a vital role in the genesis of stress-related disorders such as anxiety and depression[11,14,15]. One characteristic these disorders share is an increased emotional response to neutral stimuli[16]. Given that the amygdala mediates emotional responses to stress[12,17], it is an important structure to study in order to further understand predispositions of certain individuals to psychiatric diseases.

The amygdala receives input from sensory modalities and integrates this information to activate behavioral and physiological responses[1]. Functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies[18] in individuals with depression and PTSD show enhanced amygdala activation[19]. Furthermore, ablation studies of the amygdala demonstrate decreased fear behavior and more docile demeanor in animals[20], implicating a role for this limbic structure in fear potentiation. Among the amygdala nuclei, the CeA stands out as the major nuclei of the amygdala for three main reasons.

First, the CeA functions as the central integration point for most of the other amygdala nuclei and many other brain regions[21]. Specifically, the basolateral amygdala (BLA) receives fear memory information from the hippocampus and sends it to the CeA, causing output to regions involved in the behavioral expression of fear[22,23]. The CeA itself receives direct cortical innervations from the prefrontal cortex (PFC), sensory areas, brainstem and hypothalamus[21]. Information about the salience of danger and cognition associated with it is sent to the CeA directly from the mPFC or indirectly through the BLA[16]. This sensation of danger is heightened in individuals with anxiety disorders such that even neutral stimuli can evoke an emotional

[A] **Corticotropin Releasing Hormone (**CRH): a 41-amino acid peptide involved in modulation of neuroendocrine, autonomic, and behavioral responses to stress.

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response[16]. Noradrenergic projections from the locus coeruleus to the CeA increases CeA activity and CRH mRNA in the CeA and affects autonomic activation[24]. The information sent into the CeA is thus integrated in a process that remains to be elucidated and is expressed in the form of autonomic and behavioral responses in CeA outputs.

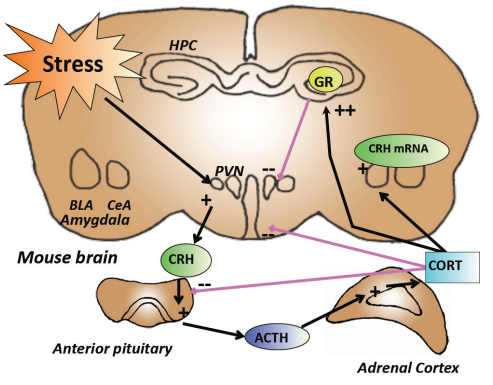

Figure 1: Hypothalamic-Pituitary-Adrenal (HPA) Axis Circuitry: Stress activation of the HPA axis as described in text leads to an increase in CRH in the central nucleus of the amygdala (CeA). GR, glucocorticoid receptor; HPC, hippocampus; BLA, basolateral amygdala; CRH, corticotropin releasing hormone; ACTH, adrenocorticotropin hormone, CORT, corticosterone.

+ activation, - inhibition

Second, the CeA is the major amygdala output structure[21] to regions involved in the stress response. Efferents of the CeA go to bed nucleus of the stria terminalis (BNST), hypothalamus, brainstem and midbrain nuclei, and modulate autonomic and behavioral functions[21]. The CeA mainly contains GABAergic output and as such, its projections to the BNST disinhibit BNST inhibition of the PVN[2,21]. Due to the limited direct CeA to PVN projections[25], this is believed to be the method by which the CeA activates the HPA axis[12], however there is no direct evidence for this. The CeA activates brainstem nuclei such as the periaqueductal gray to cause freezing and vocalization in the conditioned fear response to shock[21], and hence generates relevant fear responses to aversive stimuli[26]. In psychiatric diseases, stimuli can activate the sympathetic nervous system, possibly through CeA activation of the locus coeruleus[27], resulting in increased heart rate and blood pressure[28]. Electrical stimulation of the CeA also activates the sympathetic nervous system[21,28] and is believed to activate the HPA axis and lead to an increase in plasma CORT in rats[29].

Third, the CeA is a site containing large populations of CRH neurons[23,27] most of which colocalize with GAD65/67, implying their GABAergic inhibitory activity[27]. Moreover, electrophysiological data shows that CRH application increases GABA inhibitory post-synaptic currents (IPSCs) in the CeA[14,30]. Not only does CRH act at receptors in the CeA, but it also activates CeA targets through disinhibition of interneurons[14]. However, other studies using neuronal tract tracing methods demonstrate that CRH immunoreactive CeA neurons form excitatory synapses with locus coeruleus dendrites[31] to cause excitation of the sympathetic nervous system. This indicates that CeA CRH can function both through excitatory and inhibitory pathways to exert its function.

This circuitry of the CeA identifies a role for it in mediating pathways involved in behavioral, autonomic, and endocrine responses to stimuli[32], in part mediated by CRH. The role of CRH in affecting CeA targets may cause changes in behavior that increase risk for psychiatric disease. These roles of CeA CRH will be examined in the remainder of this review.

Role of CRH in CEA Function

The CRH peptide is distributed throughout the brain but found more concentrated in the PVN, CeA, and BnST[23,33]. CRH has two receptors, CRH-R1 and CRH-R2, which have primarily non-overlapping expressions in the brain[34,35]. CRH-R1 is more potently activated by CRH than the CRH-R2 receptors[36] and is known to be the major CRH receptor that activates the HPA axis and the stress response[35]. Restraint stress in rodents significantly increases the levels of CRH and CRH-R1 mRNA in the PVN[37]. CRH-R2 function is not clearly understood, but it is implicated in reducing stress sensitivity[38] as its deletion leads to a rise in anxiety behavior[8]. In the amygdala, CRH-R1 receptors are the predominant type expressed[35]. Functionally, inhibition of CRH-R1 receptors prevent the CRH induced increase in GABAergic IPSCs in the CeA previously described[14] and may also affect autonomic and behavioral functions of the CeA.

Studies in patients with depression and PTSD show distinct elevations in CRH levels in the cerebrospinal fluid[8,39-43]. Treatment of these depressed patients with CRH-R1 antagonist ameliorates the symptoms of anxiety and depression[44] and decreases HPA axis activity[45]. Many anxiety and despair symptoms can be recapitulated in rodent models upon intracerebroventricular CRH administration, through a pathway that is thought to be HPA axis–independent[46]. This implies that extrahypothalamic sources of CRH are the main source of CSF CRH[46]. Since CeA CRH is anxiogenic[26] and CeA stimulation exacerbates the effects of intracerebroventricular CRH[17], the CeA is a probable extrahypothalamic source of CSF CRH. This hypothesis is supported in rodent models using both in-vivo microdialysis studies that demonstrate elevated CeA CRH following restraint stress[47], as well as chronic CORT administrations. Furthermore, elevated CeA CRH in animal models also demonstrate elevated anxiety[7] suggesting that changes in CeA CRH levels are a potential model of CeA dysfunction in psychiatric disease.

Evidence for the Role of CeA CRH in Stress-Related Behavior

Numerous brain functions are sensitive to stress and can be evaluated in behavioral paradigms that have been validated over the years. Two such brain functions are learning and memory, some aspects of which are mediated by the CeA[9]. CeA lesions in rodents impair memory retention in the inhibitory avoidance task[48], the defensive burying task[49], and conditioned fear test[23]. Although there are other peptides found in the CeA, such as neuropeptide Y, neurotensin, and enkephalin[21], the role of CRH as the major HPA axis activator in the stress response, as well as activator of sympathetic nervous system[1] is the reason for its focus in this review.

When CeA CRH is reduced with the use of antisense oligonucleotide[23] or by GR deletion in the CeA (CeAGRKO)[9], memory retention in conditioned fear test is impaired. Furthermore, the CeAGRKO mice show rescue of conditioned fear behavior if intracerebroventricular CRH is administered before conditioned fear training[9]. It appears therefore that there is a homeostatic level of CRH required in the CeA for normal function and any deviation above or below this level can result in behavioral and neuroendocrine problems. CRH receptor antagonist applied to the CeA reduces elevated plus maze[50] and CRH-mediated anxiety behavior[51]. CRH-R1 antagonists diminish anxiety and HPA axis response, and increases exploratory behavior in

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primates[52]. Although the anxiety attenuating effects of CRH R1 antagonist was not observed in a study looking at novelty-suppressed feeding in rat[53], most studies to date do suggest a role for CeA CRH in anxiogenic behaviors[54], fear memory consolidation[9], as well as autonomic responses[2].

Behavioral data also shows that the CeA is involved in the psychological aspect of stress. Studies comparing the effects of the physical stress of treadmill running to psychological restraint stress show that the increase in CeA CRH throughout the restraint stress was not seen to the same extent in treadmill running rats[55]. This implies that the CeA is more a mediator of the psychological stress response and this mediation may occur through modulation by CRH[26].

Evidence from Development Studies of CRH-induced Behavioral Changes

Early life stress increases the risk of psychiatric disorders later in life. Therefore, the developmental time period that stress occurs also plays a role in the neuroendocrine and behavioral outcomes[56]. Adults who experienced some form of childhood stress show elevated basal cortisol, increases in ACTH responsiveness, and heightened emotional responses to stressful stimuli than control patients[56]. In the CSF of these patients, increased CRH was predicted by perceived stress during pre-school years but not during preteen years[56]. These models of early life stress have been recapitulated by studies of maternal deprivation in both primate and rodent models. The maternal deprivation model consists of taking the young away from the mothers a few hours a day for 2 weeks[57] and studying the behavior of the young when they reach adulthood. These studies show increased anxiety and depression behavior in rodents and primates that were maternally deprived[56,58]. In adulthood, maternally deprived primates show an increase in CSF CRH and rodents show increases in anxiety behavior[56]. Furthermore, CRH mRNA in the amygdala and hypothalamus, and CRH immunoreactivity in the median eminence are increased due to maternal deprivation[57]. This indicates that the time period during which one is exposed to a stress can affect the CRH system's response later in life. There is a need for animal models to explore temporal effects of CRH over-expression in the CeA.

These studies have laid the foundation for identifying behavioral and neuroendocrine effects of CRH in the CeA. However more research is needed using more physiologically relevant models to better understand the role CRH in the CeA plays in stress and psychiatric disorders.

Current Studies that Analyze the Effects of CRH Overexpression on Anxiety and Despair Behaviors in Rodents: Use of Lentiviral Vectors and Tetracycline-inducible Systems

There are a few rodent studies that have demonstrated the effects of CRH overexpression in anxiety and despair. Transgenic mice that overexpress CRH show reduced locomotor activity in a novel environment, which is exacerbated by social defeat stress[46]. In the elevated plus maze test, these mice spent less time in the open arm of the maze compared to the closed arm, indicating increased anxiety[46]. This anxiety response was abolished when α-helical CRH 9-41, a CRH antagonist, was injected into the intracerebroventricular region of the brain[46]. Transgenic mice that have an inducible tetracycline system[B] have also been used to demonstrate that forebrain CRH overexpression in the first three weeks of life produces anxiety-like behavior in the open field and light-dark preference test later in adulthood[59]. These mice show despair behavior in tail suspension and forced swim tests as well as increased CRH-R1 mRNA that are reversed upon treatment with antidepressant[59]. Recently, however, transgenic models have been developed that can overexpress CRH in specific brain regions to determine site-specific CRH function.

Regev *et al*. used lentiviral vectors[C] to specifically overexpress CRH in the CeA and the BNST of male mice and tested the effects of chronic expression of CRH in these regions using behavioral test for anxiety and depression[60]. The data showed that under non-stressed conditions, chronic CRH overexpression in the CeA had no effect on anxiety in the open field and light/dark preference tests[60]. However when the mice underwent 30 minutes of restraint stress before the behavioral tests, anxiety was attenuated, implying that chronic overexpression can cause habituation to a stressor[60]. In contrast, Keen-Rhinehart *et al*., also using lentiviral vectors to overexpress CRH in the CeA of female rats, showed increased anxiety, increased despair, and impaired negative feedback of HPA axis, all changes associated with stress pathology[12]. The discrepancy in these two studies may be a result of the length of over-expression time, and gender and/or species differences.

In order to combine the use of lentiviral vectors with the tetracycline inducible system, our lab has developed a transgenic mouse model[59] that will allow for spatial and temporal CRH overexpression (unpublished data). These mice have the CRH gene under the tetracycline responsive promoter[59]. This model allows for the use of stereotaxic injections of lentiviral reverse tetracycline transactivator into the brain region of interest to overexpress CRH as well as control the period of overexpression. Targeting the CeA will allow the study of specific changes that occur in this brain region upon HPA-axis dysregulation and how this affects diseases such as depression and PTSD.

Conclusion

Stress plays an important role in precipitating psychiatric disorders. One key mediator of an organism's response to stress is the HPA axis, which is associated with increased CRH mRNA levels in the CeA. The CeA receives sensory information from many brain regions and sends output to regions mediating autonomic, neuroendocrine, and behavioral responses. CeA CRH produces anxiogenic behavior in rodents and affects HPA axis and autonomic functions vital to the stress response. CRH overexpression during development as well as in adulthood can increase stress-related behavior and lead to increased risk for psychiatric diseases.

The use of tetracycline-inducible transgenic mice models to overexpress CRH during specific periods of development, in combination with stereotaxic injections of viral vectors, will allow for the determination of region-specific effects of CRH. By understanding region-specific functions of CRH in the stress response, the function of the CRH system can be elucidated to determine when a response will either cause a return to homeostasis or a drive towards psychiatric disease. Ultimately, this will enable the identification of more effective treatment of psychiatric diseases, thereby increasing human quality of life.

[B] **Tetracycline-inducible system**: Transgenic mice are generated with the gene of interest under the tetracycline responsive promoter. In the presence or absence of doxycycline, transcription can be turned on or off in these mice.

[C] **Lentiviral Vectors**: Vectors used to efficiently introduce genes into *in vivo* systems.

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    This is the first paper to investigate the use of lentiviral vectors to overexpress CRH in the CeA of mice and determine possible anxiety effects.

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Rare Variation in the Serotonin Transporter and Autism Spectrum Disorders: Examining 5-HT-mediated Effects on Neurodevelopment and Behavior

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Abstract
Elevated whole blood serotonin (5-HT) levels are present in approximately a quarter of individuals with autism spectrum disorders (ASD). There is mounting evidence that altered developmental 5-HT signaling in the brain may play a role in the etiology of ASD. In particular, the serotonin transporter (SERT), a key regulator of 5-HT homeostasis, has been repeatedly implicated in the disorder. Rare coding variants have been discovered that alter SERT function and regulation, and are associated with specific ASD features. *In vivo* modeling of rare, ASD-associated SERT mutations in mice offers an opportunity to examine how changes in 5-HT signaling during development can disrupt formation of brain circuits and cause specific ASD behavioral symptoms and traits.

Keywords: *Serotonin; Serotonin transporter; Autism spectrum disorder; Restricted, repetitive behavior; Sensory aversion; Gly56Ala; Barrel field; Thalamocortical axons; Mouse model*

ASD Background

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental disorders that share core features of impairment in social communication and interaction, as well as restricted, repetitive behavior. ASD emerges during early childhood, but may not fully manifest itself until social demands are placed on an affected individual. Recent epidemiological studies have estimated the prevalence of ASD, which includes autistic disorder, Asperger syndrome, pervasive developmental disorder not otherwise specified (PDDNOS), and childhood disintegrative disorder (CDD), to be one child in every 150 children[1]. Males are at increased risk for ASD, with an affected male to female ratio of 4:1[1]. In addition to the core diagnostic features, individuals with ASD often display secondary symptoms such as gastrointestinal dysfunction[2], epilepsy[3], and sensory aversion[4] at a much higher rate than the general population.

Among neuropsychiatric disorders, ASD has been shown to be the most heritable[5]. Depending on diagnostic criteria, family and twin studies estimate concordance rates between 60-90% for monozygotic twins compared to 0-10% for dizygotic twins[6]. Parents and siblings of affected individuals are more likely to show subtle behavioral abnormalities that mirror deficits seen in ASD[7,8]. Also, ASD is observed in subpopulations of individuals with rare genetic syndromes, such as fragile X syndrome, Rett syndrome, Angelman syndrome, and tuberous sclerosis[9]. Genetic linkage and association studies have implicated numerous candidate genes in the etiology of ASD. From these studies, functional single nucleotide polymorphisms (SNPs) and copy number variants (CNVs) have been discovered that may underlie the genetic risk. However, it is evident that ASD is a complex, polygenic disorder. To date, no single genetic variation has been implicated in more than 1-2% of ASD cases[9]. This has led some to hypothesize that the additive effects of multiple risk alleles may cause ASD and contribute to the phenotypic heterogeneity of the disorder[10]. Data emerging from genome wide association studies have yet to confirm this hypothesis, but these studies have been statistically underpowered to reliably detect alleles that confer small or moderate risk[11].

Serotonin and ASD

One of the oldest, yet most consistently replicated findings in individuals with ASD is elevated whole blood serotonin (5-hydroxytryptamine, 5-HT) or hyperserotonemia[12]. Studies have estimated that 25% of children with ASD have hyperserotonemia[13]. In the periphery, 5-HT is synthesized by enterochromaffin cells that line the gastrointestinal tract. It is then released into enteric circulation and taken up by platelets, which contain approximately 99% of 5-HT in the blood[14]. Multiple studies have indicated a positive correlation of 5-HT platelet levels between ASD cases and their parents and siblings[15,16]. In the Hutterites, a large founder population, whole blood 5-HT levels were found to be more heritable than ASD itself[17]. Since hyperserotonemia has been shown to be a reliable endophenotype[A] for ASD, there has been a great deal of interest in the genetics and molecular mechanisms that control 5-HT uptake into platelets.

Investigations of disrupted platelet 5-HT regulation in ASD have primarily focused on the integrin β3 subunit (ITGB3), 5-HT_2 receptors, and the serotonin transporter (SERT or 5-HTT). Additional linkage and association studies in the Hutterites mapped *ITGB3* and *SLC6A4*, the gene encoding SERT, as quantitative trait loci for whole blood 5-HT levels[18]. Variation in *ITGB3*[19] and a gene-gene interaction with *SLC6A4*[20] have been associated with ASD susceptibility. Furthermore, there is evidence of decreased platelet 5-HT_2 receptor binding in ASD[21]. Activation of 5-HT_{2A} receptors has been shown to regulate SERT localization in platelets[22], providing a potential mechanism that could be disrupted in hyperserotonemic ASD cases. Due to its central role in the uptake of 5-HT, the serotonin transporter has been the main focus of research into serotonergic function in ASD, and will be discussed at length later in this review.

Ultimately, peripheral 5-HT findings in ASD must be applied to the brain to understand the complex cognitive and behavioral features of the disorder. Mirroring data from platelets, human neuroimaging studies have found decreased brain 5-HT_2 receptor binding in ASD[23,24]. In addition, depletion of the amino acid tryptophan, the precursor of 5-HT, has been shown to exacerbate stereotyped behaviors in adults with ASD[25]. Tryptophan

[A] **Endophenotype**: a behavioral, cognitive, or biological measure that is a heritable trait in a psychiatric disorder.

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depletion acutely reduces serotonin synthesis, leading to a hypothetical net reduction in serotonergic neurotransmission. Interestingly, selective serotonin reuptake inhibitors (SSRIs), which have the net effect of increasing serotonergic neurotransmission, have been shown to ameliorate repetitive behavior and aggression in ASD[26,27]. Altered developmental trajectories of serotonin synthesis capacity in the brain have also been reported in the disorder[28]. These converging lines of evidence suggest that 5-HT abnormalities in the brain and the periphery play a role in the etiology of ASD.

SERT and ASD

Due to the replicated finding of hyperserotonemia as an ASD biomarker, SERT has been an attractive research target for the disorder. Similar to other monoamine transporters, SERT is an integral plasma membrane protein composed of twelve transmembrane domains, which actively transports 5-HT in an ion-coupled and anti-depressant sensitive manner[29,30]. SERT terminates 5-HT signaling by transporting 5-HT from the synaptic space back into presynaptic terminals, where the neurotransmitter can be repackaged into vesicles for rerelease[31]. ASD linkage studies have implicated the chromosome 17q11-q21 region that contains *SLC6A4*, the gene that encodes SERT[32,33]. When families with only affected males are considered, the linkage signal in *SLC6A4* region significantly increases, suggesting SERT may harbor variants that play a role in the gender bias in ASD[33,34].

The effect of common genetic variation in *SLC6A4* has been the source of much debate in neuropsychiatric research. In particular, the serotonin transporter gene linked polymorphic region (5-HTTLPR) has been scrutinized for its potential role in ASD. The 5-HTTLPR contains a 44 base pair insertion/deletion polymorphism that has a functional impact on transcription[35]. The short allele, which reduces *SLC6A4* expression, has been overtransmitted in ASD compared to controls in several studies[36,37]. However, this association has not been consistently replicated[38,39], and other functional variants in the gene further complicate 5-HTTLPR analysis. Two SNPs in the promoter region, rs25531[40] and rs25532[41], can modulate activity of the long allele and reduce *SLC6A4* expression. In addition, a variable number of tandem repeats (VNTR) polymorphism in intron 2 has also been shown to regulate *SLC6A4* expression[42]. The inconsistent associations with ASD have led some groups to speculate that common *SLC6A4* variation may modulate specific behavioral features and symptoms of ASD. Brune *et al.* found that the 5-HTTLPR short allele is associated with poor nonverbal communication in ASD, while the long allele is associated with stereotyped, restrictive motor mannerisms and aggressive behavior[43]. Also, Mulder *et al.* reported association between the intron 2 VNTR 12-repeat allele and rigid-compulsive behavior in ASD[44].

Although common genetic variants of SERT have been an area of intense focus, modest association results have not explained the suggestive linkage of the *SLC6A4* locus in ASD. To determine if multiple, rare functional variants contribute towards *SLC6A4* linkage, Sutcliffe *et al.* screened 120 multiplex families that had a significant male biased linkage signal at 17q11[34]. In their sample population, they found five rare non-synonymous coding SNPs that were overtransmitted to affected family members and were associated with rigid-compulsive behavior: Gly56Ala, Ile425Leu, Phe465Leu, Leu550Val, Lys605Asn[34]. Three of the coding variants, Ile425Leu, Phe465Leu, Leu550Val, are located within transmembrane domains at highly conserved amino acids[34]. Interestingly, Ozaki *et al*. discovered a SERT Ile425Val variant in two unrelated OCD pedigrees that had comorbidity for Asperger syndrome and other neuropsychiatric disorders[45]. The Gly56Ala variant, which will be focused upon later in this review, is located at a highly conserved amino acid residue in the intracellular N-terminus tail of SERT and was the most common rare mutation found in the Sutcliffe *et al*. study[34]. The Ala56 allele was overtransmitted 3:1 to individuals with ASD, displayed a male gender bias, and was significantly associated with sensory aversion[B] and rigid-compulsive behavior[34]. Also, initial characterization of transformed lymphoblasts from families with the SERT Ala56 allele indicated that the variant caused elevated 5-HT uptake that was insensitive to protein kinase G (PKG) and p38 mitogen activated protein kinase (p38 MAPK) pathway regulation[34]. The discovery of these rare SERT variants supports the hypothesis that allelic heterogeneity of *SLC6A4* confers risk of ASD.

Serotonin and Neurodevelopment

Although 5-HT is primarily known as a canonical neurotransmitter, there is mounting evidence that it has a pleiotropic[C] role in neurodevelopment. The early maturation of the serotonergic system initially prompted speculation that 5-HT had a developmental function. In the brain, 5-HT is exclusively produced in the rostral and caudal raphe nuclei groups (B1-B9). These serotonergic neurons are produced as early as E10-12 in rodents and begin producing 5-HT within a day of their birth[46]. In addition to endogenously produced 5-HT, maternal 5-HT can cross the placenta and fetal blood-brain barrier[47]. The rostral raphe nuclei groups (B6-B9) consist of serotonergic neurons whose axons project towards the telencephalon. Serotonergic afferents reach the telencephalon at E15, as the cortical plate begins to form[46]. Several 5-HT receptor subtypes have shown embryonic expression in a variety of brain regions[48,49]. In a landmark study demonstrating the developmental role of 5-HT signaling, Gross *et al.* found that temporally restricted deletion of 5-HT_{1A} receptors during the early postnatal period was sufficient to produce anxiety-like phenotypes in adult mice[50].

5-HT mediated neurodevelopmental processes are now beginning to be explored in greater detail. Pharmacological and genetic manipulation of 5-HT levels in the embryonic brain have implicated 5-HT as a modulator of neuronal migration. Vitalis *et al*. demonstrated that embryonic 5-HT depletion with p-chlorophenylalanine (PCPA), an inhibitor of serotonin synthesis, causes migratory and differentiation defects in specific populations of GABAergic interneurons emanating from the caudal ganglionic eminence (CGE)[51]. The authors speculate that 5-HT_3 receptors, which are expressed by CGE-derived interneurons, may regulate migration[51]. In contrast, Riccio *et al.* examined GABAergic interneuron migration defects in SERT knockout mice, which have elevated synaptic 5-HT levels[52]. Interestingly, they found that only interneurons expressing 5-HT_6 receptors were sensitive to excessive 5-HT during development[52]. Furthermore, SERT knockouts displayed altered interneuron density in primary somatosensory cortex[52], a well-documented 5-HT sensitive brain region.

The developmental impact of altered 5-HT levels has been intensely studied in rodent barrel field architecture of primary somatosensory cortex. Barrel field architecture is formed by thalamocortical axons (TCAs) synapsing with layer IV cortical neurons[53]. Each individual barrel is a cortical representation of a mystacial vibrissa , where layer IV neurons within a barrel will fire in response to a tactile stimulus applied to a particular vibrissa[53]. Removal of monoamine oxidase A (MAOA), the main enzyme responsible for 5-HT degradation, has been shown to eliminate barrel field formation due to excessive 5-HT levels during the perinatal period of development[54]. In an effort to understand how excessive 5-HT could disrupt cortical soma-

[B] **Sensory aversion**: hypersensitivity to multiple sensory modalities that causes distress.
[C] **Pleiotropic**: producing more than one effect.

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tosensory maps, Salichon *et al*. crossed MAOA knockouts with mice lacking SERT or the 5-HT_{1B} receptor[55], which are both transiently expressed in TCAs during development[56,57]. The authors determined that disruptions in barrel field formation were due to overactivation of 5-HT_{1B} receptors by increased levels of synaptic 5-HT[55].

Recently, Bonnin *et al.* further examined the molecular mechanisms involved in 5-HT regulation of thalamocortical axonal pathfinding[58]. During development, 5-HT_{1B} and 5-HT_{1D} receptors in the thalamus have overlapping expression with axon guidance receptors DCC and Unc5c[58]. Using thalamic explant cultures, they found that attractive cues exerted by netrin-1 on posterior TCAs became repulsive with activation of 5-$HT_{1B/1D}$ receptors[58]. Also, TCAs could be deflected *in vivo* using *in utero* electroporation of siRNAs and expression plasmids to alter 5-HT_{1B} and 5-HT_{1D} receptor expression in the thalamus[58]. Their data suggest that alterations in 5-HT signaling during development could cause subtle topographical shifts in TCA brain circuits. Furthermore, Esaki *et al.* have presented evidence that developmental changes in TCA circuits may have a functional impact in adulthood. They reported that SERT knockout mice, which possess disrupted barrel field architecture, have altered cerebral glucose utilization during whisker stimulation[59]. Perinatal administration of PCPA restored normal somatosensory responses in adult SERT knockouts[59]. Collectively, these studies support the hypothesis that disruptions in the serotonergic system during development could be the basis for a neurodevelopmental disorder such as ASD.

SERT Gly56Ala: Modeling ASD in Mice

As described earlier in this review, a rare SERT coding variant, Gly56Ala variant, is associated with ASD, rigid-compulsive behavior, and sensory aversion in families with evidence of linkage at the *SLC6A4* locus[34]. Characterization of lymphoblasts from ASD probands indicated that the 56Ala variant causes elevated 5-HT transport[34]. 5-HT transport was insensitive to PKG and p38 MAPK stimulation, which regulate SERT surface expression and catalytic activity, respectively[34]. Furthermore, SERT Ala56 was found to be hyperphosphorylated under basal conditions and lacks enhanced phosphorylation via PKG stimulation[60]. Additional functional studies of the Ala56 variant in transiently transfected HeLa cells have demonstrated alterations of protein kinase C (PKC) and protein phosphatase 2A (PP2A) regulation of SERT[61]. Although *in vitro* analysis of ASD-associated SERT variants has greatly aided our understanding of SERT function and regulation, *in vivo* brain studies are now necessary to understand how a given mutation impacts behavior and neurodevelopment. With this goal in mind, our lab generated a knock-in mouse line with the most common ASD-associated SERT variant, the Ala56 allele, inserted into the native mouse 129S6 *Slc6a4* gene[62].

New discoveries of rare variants strongly implicated in the etiology of ASD have spurred the development of mouse models with analogous mutations to evaluate comparable ASD phenotypes and to dissect the molecular mechanisms that underlie ASD traits. In general, effective animal models of human disorders should: 1) exhibit similar symptoms and traits found in the human disorder (face validity); 2) possess the same biological cause as the human disorder (construct validity); 3) respond to treatments that prevent or reverse symptoms of the human disorder (predicative validity)[63]. Due to the complex phenotypes exhibited in ASD, it is challenging for behavioral neuroscientists to design behavioral tasks that examine the face validity of mouse models of ASD.

This review highlights the growing library of assays to examine restricted, repetitive behavior and sensory dysfunction, two traits associated with the SERT Gly56Ala variant in humans. Mice exhibit stereotyped motor behaviors such as circling, jumping, and self-grooming that are altered in models of ASD[64]. Reversal learning tasks, such as T-maze and Morris water maze, measure a mouse's ability to switch from a learned habit to a new habit. Impairments in acquiring a new habit are thought to have face validity to 'insistence of sameness' behavior seen in individuals with ASD[64]. Disruptions in mouse exploratory behavior (i.e. locomotion, sniffing, nose poking) are also being examined as a relevant measure of restricted interests in ASD[65]. Current paradigms that evaluate sensory-related behavior in mice primarily focus on sensorimotor gating or pain tolerance. Deficits in prepulse inhibition (PPI), a measure of sensorimotor gating, have been reported in individuals with ASD[66] and offer a mouse behavioral assay with face validity to an ASD trait. In addition to sensorimotor gating, sensory dysfunction in mice is commonly evaluated by assays that measure nociceptive responses. Behavioral assays such as the hot plate or tail flick test assess thermal sensitivity in mice, but their relevance to ASD symptomology is unclear[67].

Conclusion

The diverse behavioral features and secondary traits that are observed in ASD have largely hindered research into the etiology of the neurodevelopmental disorder. The complex, polygenic origin of ASD is thought to underlie the disorder's phenotypic heterogeneity. Hyperserotonemia, a proven biomarker observed in almost a quarter of ASD cases, offers an avenue of research into the molecular mechanisms and altered brain circuits that produce specific ASD traits. The development of animal models of ASD is necessary for discovering effective treatments. As described in this review, rare variation in SERT has been highly associated with rigid-compulsive disorders in male individuals with ASD. The most common ASD-associated SERT variant, Gly56Ala, is also linked to sensory aversion. *In vivo* modeling of ASD-associated SERT variants offers a unique opportunity to study how altered 5-HT signaling during development can cause specific ASD behavioral features and traits.

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$GABA_A$ Receptor Heterogeneity at the Synapse: the α-Subunit Subtype Story

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Abstract
$GABA_A$ receptors mediate the majority of fast inhibitory neurotransmission in the adult CNS. Given its widespread role, it is not surprising that $GABA_A$ receptors have diverse subunit compositions and receptor properties to facilitate inhibitory neurotransmission in disparate neuronal networks. The functional heterogeneity of $GABA_A$ receptors is strongly dependent on the receptor's subunit composition. Of particular interest for this review are the synaptic $GABA_A$ receptor α-subunit subtypes of the adult CNS which display the highest degree of heterogeneity relative to other subunit families. Each α-subunit subtype confers unique biophysical properties and distinct temporal expression patterns to their respective receptor isoform. This review will explore how these factors contribute to $GABA_A$ receptor heterogeneity which has great implications for both $GABA_A$ receptor function, as well as, pathologies that result from impaired $GABA_A$ mediated neurotransmission, such as age-dependent epilepsy.

Key Words: *receptor heterogeneity; synaptic GABAA receptors; phasic inhibition; α-subunit subtype; epilepsy*

Introduction

GABA-A receptors ($GABA_A$Rs) are ligand-gated ion channels that mediate fast inhibitory neurotransmission in the adult central nervous system (CNS)[1]. They belong to the gene family of Cys-loop, ligand-gated ion channels (LGIC) which include other receptors such as the nicotinic acetylcholine (nAChRs) and, the glycine receptors (GlyRs)[2,3]. Similar to most members of this family, $GABA_A$ receptors are heteropentamers assembled from a large array of homologous subunits such that, when viewed top-down from the synaptic cleft (Figure 1a), the receptors are predicted to have a circular structure with individual subunits arranged pseudosymmetrically around a central ion-conducting pore (Figure 1a)[2,3]. Thus far, nineteen (19) individual subunit subtypes, grouped according to sequence homology into eight (8) subunit families, have been identified (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3)[4]. Each subunit subtype imparts unique biophysiological characteristics to their respective $GABA_A$ receptor isoforms[5-7], and exhibits distinct patterns of temporal expression dominance[8]—characteristics that will be discussed in the body of the review. Such an extensive repertoire of $GABA_A$ receptor subunits alludes to the potential for both promiscuous subunit combinations and diverse $GABA_A$ receptor properties. However, despite the relatively enormous possibilities for receptor combinations, the most predominant $GABA_A$ receptor isoform is composed of two (2) α-subunits, two (2) β-subunits and one(1) γ- or δ-subunit (Figure 1a), with the majority of receptors comprised of the γ2-subunit subtype[1], as well as a single type of α-subunit[9,10]. Nonetheless, intrinsic properties imparted by either subunit, particularly those of the α-subunit family, adequately diversify the characteristics of this predominant receptor isoform, thereby increasing $GABA_A$ receptor heterogeneity and subsequent utility within the diverse neuronal networks of the CNS[11].

The Relationship Between $GABA_A$ Receptor Function and Ligand Binding

$GABA_A$ receptor signaling in the adult nervous system is mediated by γ-aminobutyric acid (GABA), the most abundant inhibitory neurotransmitter in the mature CNS. GABA exerts its fast inhibitory effects by interacting with the $GABA_A$ receptor at two ligand binding sites, each located between the α- and β- subunit[12,13] (Figure 1a). Upon ligand binding, the $GABA_A$ receptor undergoes conformational changes, resulting in a net entry of chloride ions through the channel's pore. This net entry of anions permits a hyperpolarizing postsynaptic potential thereby reducing the probability of generating an action potential[1,14,15].

All $GABA_A$ receptor subunits share a similar structure which include: a large, extracellular N-terminus with ligand binding sites, four (4) alpha-helical transmembrane domains (TM1-4), a large cytoplasmic loop (M3-M4 loop), and an extracellular M2-M3 linker[16] (Figure 1b). These structural features are important determinants of receptor function, particularly $GABA_A$ receptor gating which represents the receptor's transition between the closed, open (ion-conducting) and desensitized (long-lived, agonist bound closed) states. $GABA_A$ receptor gating is most often induced by ligand binding in the N-terminus which is presumed to cause conformational changes within this region. Furthermore, it is believed that the ligand binding-induced conformational changes in the N-terminus are subsequently transduced down the receptor to its transmembrane domains and linker regions; thereby resulting in channel opening and $GABA_A$ receptor mediated signaling. One structural feature that contributes to efficient $GABA_A$ receptor gating is the M2-M3 linker (Figure 1b), which is suspected to couple agonist binding in the N-terminus to conformational changes in the receptor's transmembrane domains[17,18]. This coupling is believed to result from

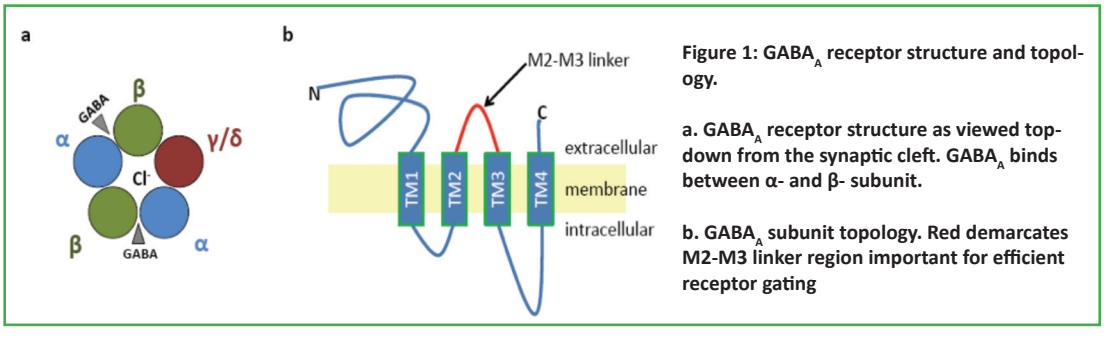

Figure 1: $GABA_A$ receptor structure and topology.

a. $GABA_A$ receptor structure as viewed top-down from the synaptic cleft. $GABA_A$ binds between α- and β- subunit.

b. $GABA_A$ subunit topology. Red demarcates M2-M3 linker region important for efficient receptor gating

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electrostatic interactions between aspartate residues within the N terminus and a highly conserved lysine residue in the M2-M3 linker region. Indeed, non-conserved amino acid changes of this lysine residue has been implicated in pathologies (e.g. epilepsy) that strongly suggest impairment of $GABA_A$ receptor gating[19-21]. Though the structural features of the receptor facilitates its cardinal properties, additional factors such as the receptor's intrinsic qualities imparted by diverse subunit compositions as well as the temporal profile of the receptor's exposure to GABA further determines the nature of $GABA_A$ mediated signaling[15].

$GABA_A$ Receptors Demonstrate Receptor Heterogeneity Through Two Types of Inhibitory Neurotransmission

As discussed in the previous paragraph, the nature of $GABA_A$ receptor neurotransmission may be influenced by both diverse receptor subunit composition and the temporal profile of receptor's exposure to GABA. Such influences are demonstrated by the two modes of $GABA_A$ mediated neurotransmission: tonic and phasic inhibition[22]. Tonic inhibition describes the continuous activation of extrasynaptic $GABA_A$ receptors by low concentrations (~1μM)[23] of ambient GABA owing to spill-over from the synaptic cleft. Given the environment within which they function, i.e. relatively far from the site of active GABA release[24], it is not surprising that these receptors have befitting biophysical properties, namely: a higher sensitivity to GABA and a significantly slower rate of receptor desensitization[A] compared to their synaptic counterparts[7,25,26]. These highly efficacious properties are largely bestowed by the δ-subunit, that with the exception of α5βxγ2 receptors, is the predominant subunit within extrasynaptic receptors[7,27,28] (Figure 1a). Furthermore, these predominant δ-subunit containing receptors are also largely comprised of the α4- or α6-subunit that additionally enhance the heterogeneity of extrasynaptic receptors in the CNS[8,26,27,29,30]. Though $GABA_A$ receptor- mediated tonic inhibition is an important component of inhibitory tone in the brain, henceforth, this review will focus on the other type of $GABA_A$ mediated neurotransmission: phasic inhibition.

Phasic Inhibitory Neurotransmission and Synaptic $GABA_A$ Receptor Function

In contrast to tonic inhibition, phasic inhibition refers to the transient activation of synaptic $GABA_A$ receptors by saturating concentrations (~1mM)[31] of GABA released from presynaptic terminals[32,33]. Consequent to GABA release, the synaptic $GABA_A$ receptors experience a rapid but short exposure to GABA that results from the ligand's rapid diffusion away from its release site[34]. This defining feature of the phasic mode of receptor activation gives rise to inhibitory postsynaptic currents (IPSCs) that activate rapidly and desensitize extensively[25] resulting in transient IPSCs. Therefore, unlike tonic inhibitory neurotransmission, phasic synaptic communication is tailored to facilitate the rapid and precise transmission of presynaptic activity into post synaptic signals.

Synaptic $GABA_A$ receptors also share a predominant subunit, namely the γ2-subunit, which confers unique biophysical properties ideally suited to mediate rapid, inhibitory neurotransmission[1]. Accordingly, γ2-containing synaptic receptors have faster activation and desensitization rates as compared to δ-containing receptors. Besides this function, γ2-subunits also play a central role in localizing these receptors at GABAergic synapses through interactions with the microtubule-associated protein, gephyrin[35,36]. Though the precise molecular interactions between the γ2-subunit and gephryin are yet to be established[37], *in vivo* experiments strongly demonstrate that gephryin plays a central role in clustering synaptic GABAA receptors[38]. In addition to gephryin stabilization of synaptic GABAA receptors, heterogeneity of receptor localization within post-synaptic densities is further increased by the receptor's α-subunit composition.

Synaptic α-Subunits and Heterogeneity of $GABA_A$ Receptor Localization

Subcellular localization of synaptic $GABA_A$ receptors is further enhanced by the α-subunit subtypes incorporated into the γ2-containing receptors. Synaptic receptors are predominantly composed of α1, α2, or α3 subunits[39,40] (Figure 1a), with the majority (~60%) of synaptic receptors comprised of the α1 subunit—a theme that will be discussed later in the review. Within the postsynaptic specialization of GABAergic synapses, α1-containing receptors are the most predominant, while the α2-containing receptors are particularly enriched within the axon initial segment (AIS) of neurons in the mature CNS[24] . In addition to distinct subcellular localizations, these α-subunit subtypes also differentially influence the biophysical properties of their respective synaptic receptors.

α-Subunit Subtypes Increase the Functional Heterogeneity of Synaptic $GABA_A$ Receptors

The heterogeneity of synaptic $GABA_A$ receptor's biophysical properties is enhanced by the α-subunit subtype composition of the receptor[5]. One biophysical property is the receptor's sensitivity to GABA, i.e., how effectively does GABA, once bound, promote $GABA_A$ receptor gating[41]. This property may be gauged by the receptor's EC50 which is a measure of the concentration of GABA that gives the half-maximal response such that receptors with a lower EC50 value have a higher sensitivity to the ligand and vice versa. For recombinant $GABA_A$ receptors, sensitivity to GABA is most strongly affected by the type of α-subunit subtype present, with α1-subunit containing synaptic receptors having the lowest EC50, the α2-containing receptors demonstrating an intermediate EC50 value and that of the α3-subunit subtype displaying the highest EC50 value[5,42,43].

Additional α-subunit-influenced biophysical properties include activation[B] and deactivation rates[C] . Regarding synaptic $GABA_A$ receptors' activation rates, experiments with recombinant receptors demonstrate the rank order to be α2<α1<α3 (Table 1), whereby α2-containing synaptic receptors have the fastest activation rate while α3 has the slowest[5,43,45]. On the other hand, deactivation rates are in the order α1<α2<α3[5,15,45] (Table 1), indicating that α2- and α3- containing receptors have prolonged currents relative to that of α1-containing receptors.

α-Subunit Subtypes Demonstrate Distinct Temporal Expression Patterns of Dominance

Remarkably, synaptic α-subunits increase GABAA receptor heterogeneity not only by contributing intrinsic biophysical properties but by also

[A] **Desensitization:** decrease in response amplitude in the continued presence of ligand.
[B] **Activation Rate:** time required for current onset to rise from 10%-90% of peak current.
[C] **Deactivation Rate**: tate of decrease in response amplitude after the removal of ligand application.

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influencing the temporal dominance of receptor isoform expression in the CNS. *In situ* hybridization and immunohisotchemical experiments in rodent models demonstrate that the most marked change in expression occurs for the α-subunit family that may result from the changing role of GABA during development[46]. As indicated previously in the review, the α1-subunit is the most predominantly expressed α-subunit in the adult CNS; however, it is minimally expressed in the developing brain. Conversely, the α2- and α3- subunits are predominantly expressed in the immature brain, with the α3-subunit demonstrating the higher expression of the two subunit subtypes[8,47]. Nevertheless, by P12 (in rodent models) the relative abundance of the developmentally predominant α2- and α3- subunits diminishes and there is a concomitant increase to dominancy in the α1-subunit expression in most brain regions[8]. Additional evidence for this age-dependent switch has also been demonstrated in nonhuman primate models[48], as well as human post mortem CNS tissue[49] where expression levels of the α-subunits mirror the age-dependent changes exhibited in rodent models, albeit with a more protracted trajectory.

These dynamic changes in the temporal dominance of α-subunit expression allude to a temporal change in composition as well as biophysical properties of $GABA_A$ receptors within the CNS. In fact, electrophysiological experiments in rodent models support this age-dependent change in dominant α-subunit receptor composition as early postnatal IPSCs display slow-deactivating currents relative to the faster deactivating IPSCs observed in the adult neurons (Table 1); indicative of a switch in predominant expression from α2-/α3- to α1-containing receptors[50-54]. Though the dominant α-subunit switching during development and the accompanied changes in GABAergic IPSCs properties are a testament to the dynamic role α-subunits play in increasing $GABA_A$ receptor heterogeneity, the functional relevance of this α-subunit-induced increased heterogeneity is not fully understood[51], particularly at the level of the neuronal network. However examining GABAergic pathologies, such as epilepsy, may assist in enumerating the role of α-subunit-induced $GABA_A$ receptor heterogeneity on neuronal network function.

Age Dependent Epilepsy: Implications of Dynamic α-Subunit Influenced $GABA_A$ Receptor Heterogeneity

Impairment of $GABA_A$ receptor function has been linked to the pathogenesis of idiopathic generalized epilepsy (IGEs)—a category of epilepsy syndromes believed to have a strong genetic underpinning[20,21,55]. Epilepsy affects approximately 0.5-1% of the general population and is defined as recurrent, unprovoked seizures that may result from lowered inhibition (disinhibition) of neuronal networks[56]; a possible consequence of $GABA_A$ receptor dysfunction. Specifically, seizures are threshold events; any point below an individual's seizure threshold can transform that individual into a seizure prone state[57]. Therefore, small changes in $GABA_A$ receptor mediated signaling, such as those induced by genetic mutations, may reduce an individual's seizure threshold and increase their probability of seizure onset. $GABA_A$ receptor mutations, such as those within the N terminus (R43Q) and the M2-M3 linker region (K289M) of the γ2-subunit (Figure 1), have been implicated in IGE pathology[19,58]. Both *in vitro* and *in vivo* experiments strongly suggest a mutation-induced disinhibition as a mechanism for epileptogenesis[20,21,59]. Furthermore, individuals with either mutation (R43Q and K289M) carry a number of phenotypes, one of which is febrile seizures (FSs). FSs are convulsions occurring during a febrile illness with an onset of six (6) months of age. However, the seizure occurrence spontaneously remit at six (6) years of age[60] and, interestingly, the individual's risk of developing epilepsy later in life (> 6 years) is only slightly greater than that of the general population[61].

The mechanism underlying this age-dependent, spontaneous remission is unknown but one hypothesis enumerates the dynamic changes in the temporal expression of $GABA_A$ receptor subunits as a possible agency for the changes in both seizure threshold and consequent seizure susceptibility[62]. As discussed in this review, members of the α- subunit family demonstrate the most dynamic temporal changes and confer distinct properties to their respective receptor isoform. Quite possibly, different α-subunit compositions may confer disparate properties to mutation containing receptors, such as those comprised of either γ2-subunit mutations discussed above. Therefore, a progressive change in $GABA_A$ receptor α- subunit composition and biophysical properties from that of α2-/α3- to that of α1- subunit subtypes may compensate for the mutation-induced receptor dysfunction; therefore providing a possible explanation for the age-dependent remission of epilepsy.

Table 1: Properties of α1-, α2-, α3- subunits containing GABAA receptors

α_x-subunit subtype	Subcellular Localization[24]	EC50 of αxβγ2 in response to GABA[5,43,45]	Rate of αxβγ2 Activation by GABA[5,45]	Rate of αxβγ2 Deactivation[5,45]	Expression levels of αxβγ2 in adult CNS[1]	Period of Predominance in CNS[45,47]
α1	Soma/dendrites	Low	Intermediate	Fast	60%	Adult/Mature
α2	AIS[1]	Intermediate	Fast	Intermediate	15-20%	Immature
α3	---	High	Slow	Slow	10-15%	Immature

[1]AIS: Axon Initial Segment; ---: no predominant area of localization

Conclusion

$GABA_A$ receptors play a major role in mediating inhibitory transmission in the CNS, which if perturbed, can contribute to pathologies such as epilepsy. Given its widespread function, it is not surprising that the properties of synaptic $GABA_A$ receptors and its subsequent inhibitory tone demonstrate heterogeneity. As discussed, the heterogeneity of $GABA_A$ receptors is increased by its subunit composition. Particularly, the α-subunit composition of the receptor influences the heterogeneity of GABAergic synapses. The relevance of this receptor heterogeneity, however, has not yet been fully elucidated. Nonetheless, pathologies such as age-dependent epilepsy, which implicate impaired $GABA_A$ mediated signaling, may offer further understating of the impact of α-subunit-induced $GABA_A$ receptor heterogeneity on inhibitory neurotransmission within neuronal networks.

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On The Cover

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Psychology, Psychiatry and the Mind-Body Problem: Going Back to the Future to Investigate the Biological Foundations of Schizophrenia

Martin J. Schmidt

Abstract

Schizophrenia is a debilitating disorder affecting approximately 1% of the population and imposing a significant burden on society. Despite its high degree of heritability, genetics alone cannot account for disease-susceptibility. It appears that an interaction between genetic and environmental factors precipitates disease onset by affecting the expression of certain genes. The most consistent evidence across patient cohorts and brain regions is a deficit in the expression of the gene encoding glutamic acid decarboxylase 67kDa (GAD67). GAD67 is the primary enzyme responsible for producing GABA, the brain's main inhibitory neurotransmitter. GABAergic interneurons are a diverse group of cells that mediate aspects of brain function via inhibitory influence on pyramidal cells and other interneurons in various brain regions. Cholecystokinin (CCK) is a molecular marker identifying a subclass of interneurons and is itself downregulated in schizophrenia. A correlation between decreased CCK and GAD67 mRNA in the same brain region suggests that CCK+ interneurons are among those that are dysfunctional in schizophrenia. CCK+ interneurons are concentrated in brain regions that are implicated in the negative and cognitive symptoms of schizophrenia which are not well managed by current antipsychotic treatments. Empirically determining the functional consequences of GAD67 downregulation in specific types of interneurons using novel mouse models will reveal differential or additive influences of these cells on aspects of behavior and hopefully translate into improved treatment options for patients suffering from schizophrenia.

Keywords: *schizophrenia, gene expression, GAD67, CCK, interneurons, mouse behavior*

Introduction

The mind-body problem has intrigued philosophers for thousands of years. At its center is the question of whether the mind is a separate, nonphysical entity or a product of bodily processes. An individual's disposition on this issue is the foundation for the way he/she views the functions of the brain and by extension, disorders of the brain. Schizophrenia is a disorder that has intrigued psychiatrists and neuroscientists for hundreds of years. Two of the people who have influenced schizophrenia research and treatment from the beginning had different interpretations of its mind-body problem relationship. Emil Kraepelin referred to it as dementia praecox, or "premature madness" linking it to other dementias, such as Alzheimer's dementia, that have defined neuropathology. He was convinced that schizophrenia was a disorder of the brain and devoted himself to looking for pathogens and/or pathology that might explain its symptoms[1]. In contrast Eugen Bleuler described it as schizophrenia, or "split mind", and believed connecting with patients individually to understand their illness was more beneficial than studying neurobiology[2]. Psychosis, a defining feature of schizophrenia, is an impairment in distinguishing reality amongst hallucinations and delusions. This presents a problem for researchers interested in mental illness. Is it possible to scientifically study "split mind" as a disorder of reality? Interestingly, a revolution in experimental psychology was taking place at about the same time Kraepelin and Bleuler were consolidating their observations. J.B. Watson detailed his displeasure with the existing study of the mind in an article published in 1913[3]. In his view, psychological processes can be studied as a science only when subjective processes of introspection, consciousness, and the mind are excluded[3]. Watson's "behaviorism" would later be extended by scientists like B.F. Skinner to incorporate those complicated "internal" processes that have quantifiable outcomes such as value judgments, motivation, and decision-making, which are now also thought to be dysfunctional in schizophrenia. No causal pathology exists for schizophrenia[4]. However, alterations in neural connectivity and gene expression are being identified and advances in molecular biology have made it possible to mimic these insults in genetic mouse models. Although we will never recapitulate psychosis in a mouse, incorporating classical views of schizophrenia and behavior with modern molecular biology allows for the empirical analysis of molecular genetic dysfunction and its effect on the brain and on behaviors associated with complex human disorders like schizophrenia.

Schizophrenia

Schizophrenia is a debilitating disorder affecting approximately 1% of the population[5]. Symptoms fall into three domains: positive symptoms including hallucinations and delusions, negative symptoms including social withdrawal, anhedonia, avolition, etc. and cognitive symptoms including deficits in working memory, disorganized thought, and attention. In addition to the behavioral impact, cardiovascular disease and metabolic syndromes, including weight gain and diabetes among others, contribute to a mortality rate that is 2.5 times higher in schizophrenia than the general population[6-8]. Perhaps more alarming is that schizophrenics are 13 times more likely to commit suicide, adding to the already devastating emotional impact on the families of those afflicted[7]. Patients typically first experience symptoms in adolescence or early adulthood and as many as 60% experience impairment throughout life[9]. The duration and severity of the illness represent a significant financial and emotional burden to the patient, his/her family, the healthcare system, and society at large. In 2002, schizophrenia cost the United States an estimated $62.7 billion in direct healthcare costs, alternative housing, and lost productivity[10]. Adding to the negative impact on society and the healthcare of the individual, schizophrenics make up a large proportion of the homeless population, albeit difficult to quantify[10,11]. The financial and emotional toll of the illness reaches from patient to population and working towards a better understanding of its cause(s) and the biological basis of its dysfunction will enable more effective treatments to alleviate that strain.

While the exact cause(s) of schizophrenia remain elusive, heredity was quickly identified as a major factor and Kraepelin devoted a section of his early textbook to the topic[12]. Family studies have revealed a 46% risk for diagnosis of a monozygotic (identical) twin when the other is affected

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and nearly the same numbers for children with two schizophrenic parents[13]. The risk for a dizygotic (non-identical) twin is 17% when the other is diagnosed which is similar to the risk for children with one schizophrenic parent[13]. Monozygotic twins are genetically identical and would therefore share 100% of the risk if it was a purely genetic disease[13]. It is now appreciated that genetics may confer schizophrenia susceptibility while a combination of multiple factors including environmental influences may be necessary for disease manifestation[14,15]. "Environment" is a broad term that can refer to the internal surroundings that affect a particular cell and/or the external surroundings affecting an organism. Environmental factors exert this influence directly by affecting specific cellular processes (i.e. toxins) or indirectly by manipulating the expression of genes (i.e. hormones, drugs, immune system activation, etc.)[16]. This interaction between genetics and environment, through which a genetic predisposition is revealed, can explain how individuals with identical genetics (i.e. monozygotic twins) differ in subtle aspects of their appearance or personality and in some cases in drastic aspects of their physical and mental health. In the absence of gross pathology seen in other neuropsychiatric disorders[4,17], many researchers have focused their attention on the role that gene expression plays in the disease process of schizophrenia.

GABA-Associated Deficits in Schizophrenia

The involvement of GABA dysfunction in schizophrenia was established as a theoretical possibility in the 1970s based on data from animal models regarding GABAergic control of dopamine release in striatal and mesolimbic circuits[18]. The following decade foreshadowed the emergence of GABA-associated deficits as a major factor in schizophrenia with findings implicating reduced GABA content in the amygdala[19], suggesting deficient synthesis; radioligand-binding studies suggesting increased GABA receptor[20,21] and decreased GABA transporter[22] protein levels; and reduced interneuron densities[23] in post-mortem brain tissue from patients with schizophrenia. In 1995, Akbarian and colleagues published the first studies of gene expression in post-mortem schizophrenic brain tissue and found a decrease in GAD67 mRNA in prefrontal cortex that could not be accounted for by cell loss[17]. GAD67, or glutamic acid decarboxylase 67kDa, is the enzyme primarily responsible for the production of GABA in the brain[24]. The GAD67 expression deficit has become one of the most consistently replicated gene expression findings in schizophrenia across many different brain regions, patient cohorts, methods, and investigators[25-31] which is remarkable given the diverse genetics and presentation of symptoms seen in schizophrenia. Subsequent efforts have focused on illuminating the impact of GAD67 downregulation on other cell signaling pathways already implicated in schizophrenia, on the function of the brain, and ultimately on behavior.

How might GAD67 gene expression deficiency occur in schizophrenia and how might it fit with what is known about the neurobiology of schizophrenia? Interestingly, data from animal models suggest that GAD67 expression can be reduced by chronic dopamine D2-receptor stimulation[32,33] or with acute NMDA receptor antagonism[34] in multiple brain regions. These data mirror the ability of chronic dopamine stimulation[35] and acute NMDAR antagonism[36,37] to precipitate psychosis in humans. Thus, the NMDA hypofunction hypothesis, the dopamine hypothesis, and the GABA dysfunction hypothesis of schizophrenia could be integrated with GAD67 deficiency being a key player in each[29]. In addition, studies of the gene that encodes GAD67, GAD1, have yielded a number of single nucleotide polymorphisms (SNPs) that are found more frequently in schizophrenic patients than in normal individuals[38-40]. The majority of SNPs in each study was found in gene regulatory sequences not coding parts of the gene, suggesting a role in gene expression and not protein function. An analysis of patients with one of the genetic variants confirmed decreased GAD67 mRNA levels post-mortem[38]. A third mechanism that may contribute to decreased GAD67 expression in schizophrenia is epigenetics. Genes can be downregulated when promoters or other regulatory sequences are methylated, causing changes in chromatin structure that prevent transcription[41]. Methylation is carried out by enzymes called DNA methyl transferases. One such enzyme, DNMT1, is overexpressed in GABAergic interneurons of schizophrenic patients[41] correlating with a GAD67 mRNA decrease in the same cells[42]. Huang *et al.*, linked those findings by reporting an 8-fold increase in the methylation of the GAD67 gene promoter in schizophrenic patients[43]. Even more interesting is that nicotine has been shown to reverse the methylation status of the GAD67 promoter which may explain in part the high incidence of cigarette smoking among individuals with schizophrenia[44]. The fact that several different mechanisms can lead to a decrease in GAD67 gene expression illustrates how diverse genetic and environmental insults can affect the brain through a common mechanism and might explain a portion of the complexity of schizophrenia etiology.

CCK+ GABAergic Interneurons, Schizophrenia, and Behavior

At about the same time Kraepelin and Bleuler were defining schizophrenia, Santiago Ramón y Cajal was astounded by the "prodigious abundance and unusual wealth of forms of the so-called neurons with short axon"[45]. GABAergic interneurons are so diverse in fact that creating a nomenclature for their numerous defining characteristics continues to be a tedious task[46]. It is now appreciated that interneurons can be grouped based on morphological, molecular, and physiological properties[46]. Classification is important because subtypes of interneurons are involved in facilitating different processes in the brain and are concentrated in brain regions that mediate different behaviors. Therefore dysfunction of particular classes of interneurons could generate diverse pathophysiology and behavior. Cholecystokinin (CCK) is a so-called brain-gut peptide that identifies a particular class of interneurons found primarily in limbic and frontal circuits[47-49]. A correlation between decreased CCK and GAD67 mRNA in the same brain region suggests that CCK+ interneurons are among those that are dysfunctional in schizophrenia[50]. They are defined by morphology as either large multipolar/basket cells or small bipolar cells[51]. These two subclasses are further divided based on electrophysiological properties[52] highlighting the diversity within this class of interneuron. Functionally, they contribute to gamma oscillations[53,54]. Neural oscillations are the coordinated firing of neurons in particular brain regions and define the signal-to-noise ratio of neural communication[55]. Gamma oscillations are a particular type that is disrupted in schizophrenia and may underlie learning and memory deficits seen in patients[25]. Distinct from other interneuron types, CCK+ interneurons express M3 muscarinic receptors[56] and alpha7 nicotinic receptors[57] further linking them to cognitive symptoms of schizophrenia since both are promising new clinical targets for cognitive improvement[58,59]. They also express the endocannabinoid receptor CB1[57,60] which has been linked to psychosis onset and outcome[61] and the $5HT_3$ receptor which may be involved in providing "emotional, motivational, or other state dependent tuning" in a fast, temporally bound manner[62]. The role CCK+ interneurons play in behavior has yet to be assessed directly. Defining this role will contribute to an improving understanding of the biology of schizophrenia and direct treatments, like the M3 and alpha7 drugs, towards particular types of symptoms in individual patients.

When one considers the role CCK+ interneuron dysfunction plays in schizophrenia pathophysiology, brain regions concentrated with CCK+ interneurons are naturally implicated. CCK+ cells are found primarily in medial frontal cortex including limbic, orbitofrontal, and anterior cingulate

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cortices and in the amygdala and hippocampus indicating a similar concentration of CCK+ interneurons by proxy[63] although there is additional low-level CCK expression in some pyramidal cells[47,51]. This distribution is remarkably similar in mice[47], nonhuman primates[63], and humans[48,49]. These regions are linked functionally through their participation in motivation, reward, social behavior, decision-making, self-regulation, affect, and learning and memory: also remarkably similar in rodents, nonhuman primates, and humans[64,65]. In fact, many of these processes have been studied most extensively using rodents as an experimental model. Psychiatric research has begun to mirror these studies, providing evidence of dysfunctional amygdalocortical and corticolimbic circuits in psychiatric illnesses including schizophrenia[66]. Basolateral amygdala (BLA) interactions with orbitofrontal (OFC) cortices encode the value of stimuli in a dynamic way[64,67]. The OFC uses this information for decision-making along with other frontal cortical structures like the anterior cingulate (ACC). Continuous updating of these representations is necessary for adaptive learning and cognition[68]. The cortical and amygdalar contributions to this behavior are dysfunctional in schizophrenic patients[69,70] in a manner that can be reproduced in mice[65]. CCK+ interneurons may be involved in coordinating this behavior since they represent a large percentage of the interneurons in the BLA[71] and in layer II/III of medial frontal cortex[63] where BLA afferents project[72]. This situation has a clear connection to clinical data correlating decreased CCK levels with negative symptoms[73] and theoretically with cognitive symptoms. Although it is not clear if peptide expression indicates interneuron function per se, other evidence linking CCK+ interneurons specifically in the integration of the "emotional, motivational and general physiological state of the animal"[74] supports a role for dysfunction of these cells at the interface of negative and cognitive symptoms of schizophrenia.

Modeling Schizophrenia in Mice

Mouse models offer the ability to study the causal influence of genetic and environmental manipulations and their interactions on cellular, molecular, and behavioral processes that are dysfunctional in schizophrenia. Discussed here are dysfunctional GABAergic processes. An important caveat when comparing mouse and human interneurons, besides obvious differences in cortical size, is that the proportion of interneurons in the human cortex is much greater than in the mouse[75]. However, this discrepancy may also lend itself to the interpretation that findings of interneuron studies in mice could have a much more robust effect in primates. On the behavioral level, psychosis is excluded from these studies because hallucinations, delusions, disorganized speech, and thought disorder are not quantifiable in mice regardless of their validity or lack thereof. That limitation does not diminish the utility of the mouse since negative and cognitive symptoms can be modeled and represent a tremendous clinical need as they are consistent predictors of prognosis and are not well managed with current medications[76,77]. The neurobiology and neuropsychology of dysfunction in these symptom domains is beginning to merge with longstanding behavioral neuroscience research into the neural basis of normal social, affective and cognitive processes[64,67,69]. Furthermore, the neural dysfunction responsible for positive symptoms is becoming better understood[78-82]. As a result, we may be able to employ mouse models in the near future to interrogate the underlying circuitry molecularly and physiologically even if we cannot directly measure analogous behaviors. After all, the goal is not to manufacture murine schizophrenia, but to gain knowledge of the ways dysfunctional circuitry changes the behavior of an organism, however that may be, and use that knowledge to guide hypotheses that can be taken back to the clinic.

Conclusion

There are many current hypotheses of schizophrenia and even more candidate genes. It is important to note that these hypotheses are not mutually exclusive. Bleuler noted the diversity of presentation of the illness from the beginning, naming the disorder not schizophrenia, but the "group of schizophrenias" in 1911[83]. Genetic susceptibility to schizophrenia is conferred by a large number of genes that may interact with environmental factors in a number of possible combinations[15] to produce pathophysiology and symptoms. This complexity contributes to the difficulty in elucidating the neurobiology of the illness. Converging evidence is beginning to identify certain pathophysiological processes that represent the final common pathway(s) of the disorder. Experimentally defining the manifestation of dysfunction in these pathways will be the next step towards understanding how the pieces fit together to form a complex and severe mental illness. Our laboratory has developed novel mouse models that downregulate GAD67 in specific classes of interneurons[84] which will contribute to an empirical understanding of how each class may participate in the pathophysiology and behavior of schizophrenia or to particular symptom domains. These studies join a movement towards clinical research that will be more amenable to translation than qualitative descriptors of symptoms[85]. Advancing research in these areas seems to have moved the field "back to the future" of psychiatry and experimental psychology by revisiting Kraepelin's search for the biology of psychosis[1] and Watson's exclusion of the "mind" to study behavior scientifically[86,87]. While it is impossible to fully recreate schizophrenia in an animal model, the combination of a Kraepelinin search for biological underpinnings of psychiatric illness and the application of a (modern) behaviorist point-of-view to defining symptoms of the illness will permit a more thorough comparison of advanced genetic models and permit their use to develop better treatments for psychiatric illness: exactly as Kraepelin himself suggested nearly a century ago.

"However little it may be possible to identify human with animal brain-functions and illnesses, yet, from the effects produced by particular noxae in the brains of animals, conclusions can be drawn as to the issue of like processes in man." –E.Kraepelin, 1919[1]

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Induced pluripotent stem cells to model gene-environment interactions in Huntington's disease

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Abstract
Huntington's disease (HD) is a neurodegenerative disorder with hyperkinetic symptoms due to loss of medium spiny neurons (MSNs) in the caudate and putamen of the striatum. HD is caused by a polyglutamine tract expansion with longer expansions leading to earlier age at onset (AO). However, a large AO variability still exists between patients with the same size expansion. Environmental modifiers have been shown to play a large role in AO variability. Few gene-environment interactions have been studied for HD partly due to the difficulty in performing such studies in animal models. Induced pluripotent stem cells (iPSCs) can be generated from a patient's dermal cells and can be subsequently differentiated into neuronal cells. Directed differentiation of human embryonic stem cells has already been shown to generate medium spiny neurons (MSNs), the cells selectively degenerated in HD, in culture. Having a supply of HD patient-specific medium spiny neurons will allow for gene-environment interaction screening in cells containing nearly an identical expression profile and genetic background to *in vivo* patient cells. Clinical applications can then be developed, which could potentially delay AO and decrease disease severity.

Keywords: *Huntington's disease, gene-environment interactions, induced-pluripotent stem cells, directed differentiation, medium spiny neurons*

Huntington's Disease

Huntington's disease (HD) is a dominantly inherited genetic disorder that affects approximately 6 persons per 100,000[1]. A "dance-like" hyper-kinetic symptom known as chorea, one chief characteristic in the diagnosis of HD, was first accurately described by George Huntington in 1872[2]. The motor and behavioral phenotypes of this disease are caused by neurodegeneration in many brain areas but most notably the loss of medium spiny neurons found in the caudate and putamen of the striatum[3]. More recent MRI studies have found varying degrees of degeneration in nearly all brain regions in early disease[4].

In 1993, the genetic cause of the disease was narrowed to the *IT-15* gene, which encodes for a protein now known as huntingtin[5]. HD patients have an expansion in the glutamine repeat region (polyQ) in the huntingtin gene (*HTT*). Huntingtin is expressed in all tissues and is necessary for embryonic and neural development[6-8]. Huntingtin has many diverse functions, and for this reason, the mutant form leads to many diverse cellular pathologies including calcium signaling abnormalities, mitochondrial dysfunction, neurotrophic factor reduction, excitotoxicity, transcriptional dysregulation, protein aggregate formation, and altered autophagy[3,9-13]. Currently, no treatments have been proven effective for HD patients, and the specific mechanism underlying the selective degeneration is undetermined. HD occurs in individuals with repeat lengths greater than 35 glutamine codons (CAG). Individuals with 36-39 repeats show incomplete penetrance and those with >70 repeats typically display HD symptoms in childhood[14]. However, one study reported a patient with a post-mortem diagnosis of HD had a repeat length of 29 on the longer allele[15]. This observation hints toward more complexity in pathogenesis than merely repeat length.

Modifiers of HD Age at Onset

Although repeat length of the polyglutamine tract has a strong inverse relationship to the age at onset (AO)[16], additional genetic and environmental factors play a critical role. Association studies using large HD patient cohorts have attributed 50-72% of the AO variability to the length of the polyQ repeat[8,16,17]. Environmental modifiers account for 60% of the remaining AO variability[17]. This gene-environment modifier contribution to AO is thought to increase with shorter repeat length causing a larger AO variability. In fact, patients with a repeat length of 40 CAG can have an AO between 32-69 years of age[17]. Even monozygotic twins with HD have shown differences in both rate of disease progression and symptomatic manifestation despite identical genetic background[18-21]. These data taken together provide evidence that environmental modifiers play a large role in HD disease progression.

Few environmental modifiers have been found for HD, though environmental enrichment in transgenic rodent models is one robust HD modifier. Rodents with mutant huntingtin provided with running wheels and novel objects showed decreased disease severity compared to rodents not exposed to an enriched environment[22]. Reduced production and trafficking of brain-derived neurotrophic factor (BDNF) is a major phenotype of HD, and enrichment has been linked to increases in BDNF expression[13,23,24]. Dietary restriction has also been shown to increase BDNF levels in wildtype mice, and essential fatty acid supplementation prevents some motor deficits in an HD mouse model[25,26]. Environmental factors can therefore have a therapeutic role in HD.

In addition to these protective effects of environment, many environmental compounds can synergistically increase the progression of neurodegeneration. In some neurodegenerative diseases, such as Parkinson's disease (PD), environmental toxins are thought to be a primary causative agent[27]. Metals are known to increase the progression of many neurodegenerative disorders. Small concentrations of divalent metals (Fe^{2+}, Mn^{2+}, Pb^{2+}, Cu^{2+}, etc.) can greatly increase oxidative stress, a major disease mechanism of many neurodegenerative diseases[28]. Some of these metals such as iron(II) and copper(II) are known to convert superoxide and hydrogen peroxide molecules into more reactive hydroxyl radicals that can damage proteins, lipids, and DNA[28]. Mitochondrial dysfunction can also be caused by oxidative stress and is another HD disease mechanism[12].

Many neurodegenerative diseases involve altered metal ion homeostasis. Copper (II) and iron (II) are found to have increased accumulation in brains of Alzheimer's, PD, and HD patients and mouse models[28-31]. Copper can also inhibit lactate dehydrogenase leading to reduced metabolic function and accumulation of lactate, which are two phenotypes of HD[30]. Wilson's disease, which involves the accumulation of copper, often leads to

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HD-like striatal degeneration, and manganism, an overexposure to manganese, has Parkinsonian-like symptoms[30,32]. One study previously reported a decrease in manganese (II) levels in mouse striatal cells due to mutant huntingtin expression[33]. Reduced Mn^{2+} concentration was also shown to selectively occur in the striatum of an HD mouse model[33]. Many enzymes for metabolism (e.g. pyruvate decarboxylase, glutamine synthatase, and arginase) and antioxidation (e.g. SOD1 and SOD2) need metal cofactors. Therefore, both excess and insufficient concentrations of certain metal ions can also lead to reduced metabolic function and increase oxidative stress. The overlapping characteristics between metal toxicity and neurodegenerative disease as well as altered metal ion homeostasis in many neurodegenerative diseases provides a firm foundation for studying gene-environment interactions between mutant htt and metal ions.

Previous HD Models

Animal models have provided many important insights into HD disease mechanisms; however, a complementary human-based model would overcome several obstacles in HD research[34]. Model systems have included 3-nitroproprionic acid lesioned rodents transgenic mice, rats, fish, flies, and monkeys as well as immortalized murine striatal cell culture[35-37]. The divergent evolutionary time between rodents and humans has allowed for large genetic differences, and these interspecies genetic differences could alter disease mechanisms and gene-environment interactions. Furthermore, murine HD models with similar size polyQ repeats can have completely different phenotypes[3]. Interestingly, the endogenous murine huntingtin only has a polyQ expansion of 7 CAG repeats while the human gene averages around 20, and the functional significance behind this difference could also alter disease mechanisms and environmental disease modification based on species[3].

Using human cells and tissues would circumvent these genetic difference issues. Post-mortem tissues have provided an important supply of human HD culture material; however, since HD patients lose the majority of their striatal volume during disease progression, these tissues are even more limited. Peripheral tissues such as dermal fibroblasts can be propagated in culture for a limited time period, but differences in expression pattern between CNS and dermal cells reduce their usefulness for modeling neurodegeneration.

Induced Pluripotent Stem Cells

Until recently, post-mortem and peripheral tissues were the only human models available for neurodegenerative studies, but with the advent of induced pluripotent stem cell (iPSC) technology, this paradigm is changing. In 2007, Shinya Yamanaka's lab first demonstrated the direct reprogramming the epigenetic status of human somatic cells into iPSCs[39,40]. In this landmark study, four transciption factors (OCT4, KLF4, SOX2, and c-MYC) were introduced using retroviral integration into cultured human dermal fibroblasts. Within 30 days, iPSC colonies appeared in culture[41]. After establishing these colonies into stable cells lines, the endogenous pluripotency genes were found to be epigenetically active and the retroviral constructs were silenced. Further studies have validated that the expression pattern, telomerase activity, mitochondrial regulation, and pluripotency of hiPSCs is similar to that of human embryonic stem cells (hESCs)[40,42,43].

Like hESCs, iPSCs can be differentiated into any cell type in the body. Patient-specific iPSCs have also been generated (e.g. HD, ALS, PD, Down syndrome, spinal muscular atrophy) providing researchers with patient-specific cell lines for *in vitro* disease modeling (Figure 1)[44-47]. Recent studies have also provided increased reprogramming efficiencies by small molecule incubation and integration-free iPSC induction techniques using cell permeable proteins and non-integrating DNA vectors[48-50]. A recent study has also shown induction of iPSCs from culturing T cells found in a single milliliter of blood[51]. These technical advancements will soon provide a simple, integration-free patient-specific generation method for HD iPSC lines that can be differentiated into relevant cell types.

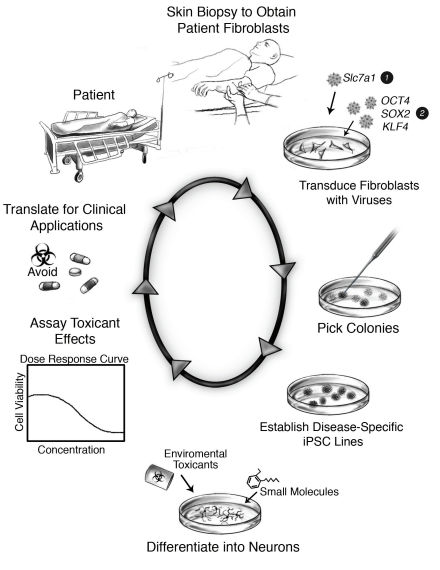

Fig 1. A schematic representation of iPSC neurodegenerative disease modeling

Neuronal Differentiation

With the correct incubation of signaling molecules, HD iPSC lines can then be differentiated into striatal neurons. Neurodevelopment requires many spatial-temporal signaling events that are complicated by the multiple roles of signaling molecules. Unfortunately, the majority of neurodevelopmental research has been based on murine embryo brain development, and the timing and function of human homologs cannot be assumed from these data. Using human pluripotent stem cells *in vitro* allows for elucidation of both normal and disordered human neurodevelopment. Several laboratories have already used human recombinant signaling proteins and small molecules to exogenously direct differentiation of iPSC and ESC *in vitro* cell cultures to enriched populations of motor neurons, midbrain dopaminergic neurons, and forebrain GABAergic and glutamatergic neurons[45,46,52-56]. Neurons differentiated from HD and control iPSC could be used for *in vitro* studies of disease mechanisms, gene-environment interactions, drug-screening, and regenerative medicine.

The first stage of human stem cell differentiation is toward one of the three germ layers: neuroectoderm, mesoderm, and endoderm. Neuroectoderm has been found to be the default tissue fate of epiblast culture, and cultured hESCs have been shown to be in an epiblast stage[57,58]. Signaling by the TGF-β super family, including bone morphogenic proteins (BMPs), inhibits the formation of neuroectoderm[59]. *In vivo*, proteins like noggin bind to BMPs to block their signaling allowing default neural tissue to form. Initial neural induction studies used either the formation of embryoid bodies or coculture with stromal cells. Unfortunately, embryoid bodies induce small numbers of neural cells, and stromal cells take several weeks to cause efficient neural induction followed by laborious mechanical picking of neural rosettes[60]. These difficulties were overcome when Chambers *et al.* induced ~80% PAX6-expressing cells, a neuroectodermal markers, in less than a week in monolayer hESC culture[53,61]. This was achieved using noggin and a TGFβ small

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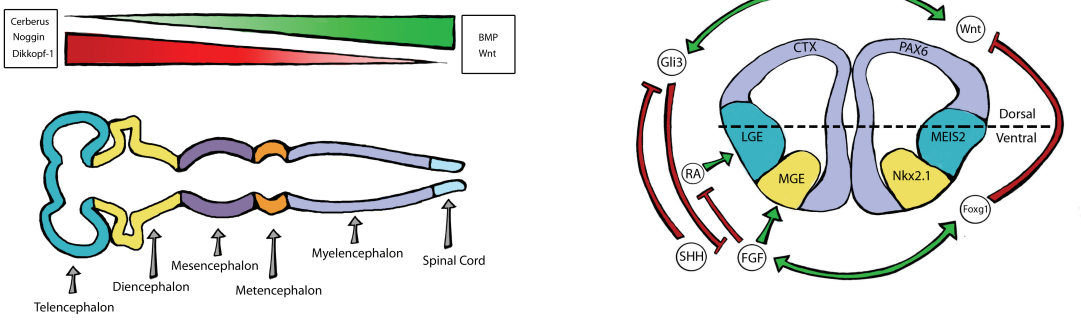

Fig 2. ***(A)*** **Several important signaling molecules in anterior-posterior axis formation. Wnt and BMP promote posterior formation, and Cerberus, noggin, and dikkopf-1 inhibit the function of these posteriorizing molcules leading to anterior formation.** ***(B)*** **Major telencephalic signaling molecules. The three major regions are labeled on the left cortex (CTX), lateral ganglionic eminence (LGE), and medial ganglionic eminence (MGE). To the right are specific markers of these regions. Sonic hedgehog (SHH), fibroblast growth factors (FGF), retinoic acid (RA), Wnt, Foxg1, and Gli3 have both active and repressive roles in setting up the dorso-ventral and medial-lateral axes.**

molecule inhibitor[53]. Noggin can also be replaced with dorsomorphin, a small molecule that blocks the downstream Alk-Smad pathway of BMP signaling[53,55]. Unfortunately, iPSC lines have been found to be highly variable in their neural induction efficiencies[62]. However, this variability may be attenuated with the production of iPSC lines screened by more strict validation methods. In any case, a large percentage of neural progenitors can be produced from iPSC lines.

Medium spiny neurons (MSNs), which are particularly vulnerable in HD, develop in the lateral ganglionic eminence of the ventral telencephalon. Gradients of signaling molecules give developing progenitors positional identity. LGE progenitors need to be patterned by signaling molecules that lead to an anterior, ventral, and finally lateral position during the neural patterning process. The anterior-posterior axis is set up by anterior expression of Wnt inhibitors, Cerberus (also a BMP inhibitor) and dikkopf-1 (DKK1), and BMP inhibitors inducing anterior fate (Figure 2A)[63]. Sonic hedgehog (SHH), is secreted from the floorplate of the neural tube, setting up the dorso-ventral axis by inhibiting the dorsally expressed Gli3 repression of fibroblast growth factor (FGF) signaling (Figure 2B)[64]. FGF is the major ventralizing signal of the CNS. FGF receptor knockouts lose most of the ventral region of the telenchepalon64. However, incubation of chick lateral ganglionic eminence (LGE) explants, which normally develops into striatum, with FGF8 causes expression of medial ganglionic eminence (MGE) markers[65]. Several laboratories have incubated hESCs with exogenous DKK-1 and SHH to produce cells expressing ventral telencephalic markers[52,54].

To specify LGE identity over MGE requires the signaling molecule retinoic acid (RA)[65]. RA expression in chick embryos causes expansion of the LGE at the expense of both MGE and dorsal regions[66]. Additional studies have found RA receptor antagonists block the formation of LGE[65]. Few studies have used RA for the specification of LGE-like progenitors despite its apparent necessity. The ventral telencephalon has been shown to endogenously produce RA, which could lead to LGE patterning *in vitro*[67,68]. However, for a more robust directed differentiation protocol, RA will almost certainly be necessary.

After patterning, neural progenitors must be induced to terminally differentiate into mature neurons. BDNF is a key molecule in striatal differentiation. It induces the expression of DARPP-32, and its loss decreases striatal projection neuron markers and MSN dendritic arborization complexity[69-71]. Additionally, exogenous expression of BDNF and noggin in the adult striatal ventricular zone has been found to reactivate nascent progenitors to become new MSN's[72]. The only report of DARPP-32 positive MSN generation from human ES cells used a combination of BDNF with valproic acid and dibutryl-cyclic-AMP, two compounds known to increase striatal neurogenesis[52,69,73]. This paper from the Perrier lab showed that MSN-like differentiation is possible; however, the protocol was highly inefficient and took nearly two months to complete. More refined protocols will be needed to generate sufficient quantities of MSNs and provide insights into HD striatal developmental since abnormalities have been reported in HD mice[74].

Modeling Disease with Patient-Specific iPSC Derived Neurons

Neurons generated from HD iPSCs must demonstrate recapitulation of an HD phenotype. Several disease-specific iPSC lines have been generated, but only a few pioneering studies have investigated phenotypes in iPSC models of neurological disease. Using iPSCs from a spinal musclar atrophy (SMA) patient and control, Ebert *et al.* reported deficits in SMN protein in an the iPS cell state[46]. This recapitulated deficits seen in the patient's dermal fibroblasts. This study also reported reduced motor neuron differentiation in the SMA mutant iPSC line. This difference is potentially due to the variability of potency between the two lines since only a single clonal line of each genotype was used[62]. Alternatively, viral construct integration could have inactivated genes altering the properties of the cell line. These issues can be mitigated in the short term by using multiple clonal lines, which will have different integration sites, and in the long term by utilizing integration-free iPS induction methods.

The Studer lab used more robust methodology in modeling familial dysautonomia (FD). Two clonal iPSC lines each from an affected and a control individual were differentiated into several tissue types, and the lowest levels of the affected transcript were found in neural crest progenitors. This correlates with the phenotype of peripheral autonomic and sensory degeneration seen in FD patients[47]. Similar genotype-phenotype interactions have not yet been demonstrated in a CNS neurodegenerative disease iPSC model.

Establishing a genotype-phenotype correlation will give validity to an HD iPSC model. Testing for 3-NP selective vulnerability in the differentiated HD iPSC lines would recapitulate phenotypes seen in current HD models[33,35]. Similar toxicant treatments have been performed on hES-dervied dopaminergic neurons; however, hES lines are not amenable to producing disease-specific lines[75]. Comparisons between disease-specific and controls cells are now possible with iPSC technology.

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Conclusion

Induced pluripotent stem cells will provide an important model for the study of Huntington's disease. These cells can be generated from patients with well-documented genotypes and symptoms in much less time than generating a transgenic mouse. They can be propagated indefinitely and differentiated into medium spiny neurons. *In vitro* differentiation allows for better understanding of human neurodevelopment and how disease and toxicants affect developmental time points. Maintenance of the human genetic background allows for an adequate model to study gene-environment interactions, and these studies could lead to treatments that delay disease onset and progression for HD patients.

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