VANDERBILT REVIEWS | NEUROSCIENCE

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Vanderbilt Brain Institute

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VRN Reviews Neuroscience at Vanderbilt

The last three years have witnessed a huge change in the administration of our Vanderbilt Brain Institute: from founder Elaine Sanders-Bush's retirement as Director, to Randy Blakely's interim directorship and pseudo-co-appointment as Director of Graduate Studies in Neuroscience, to Lou DeFilice's appointment as full DGS until his departure this past fall, to the selection of Mark Wallace as our permanent VBI Director and his appointment of Doug McMahon—whose primary appointment is in the College of Arts and Sciences—to our current DGS, and finally the departure of Assistant Director for Education Mary Early-Zald this very month. Were you able to follow? Don't worry, the Institute and its core Program are and always have been in good and capable hands. Chalk it up to growing pains in a young program. These changes are expected, or even essential in taking a very good program and making it great. But we should not be content to think that the Vanderbilt Brain Institute and its component programs are collectively *one of the best* in the nation, rather we must strive to be *the best*. To this end, we must realize that our program is beginning to enter a middle-age of sorts: no longer can the sole focus be on graduate education—financial development, community involvement and intra-/inter-collegiate collaboration must play a more prominent role in furtherance of the VBI's mission. Moreover, being *the best* does not consist solely of publications, lab funding and academic achievement—instead, we must strive to find novel and exciting ways to consolidate the greater Vanderbilt Neuroscience community and have our voices heard.

This journal, appropriately dubbed *Vanderbilt Reviews Neuroscience*, is hopefully a step in that direction. The idea came from many sources. First, there are the prestigious law journals that rule legal academics. As a highly motivated student at any given law school in the country, you would likely wish to publish your work in an exalted journal like the *Yale Law Review*, or the *Harvard Law Review* or even the *Vanderbilt Law Review*. Institutionally published journals are not only common in legal academia, they are the norm. Why isn't scientific publishing the same way? That question brings me to inspirational source #2: four years ago, two enterprising Vanderbilt undergraduates named Warren Langevin `07 and Noah Clemons `05 founded the *Vanderbilt Undergraduate Research Journal* (*VURJ*) as an open-source mechanism for anybody at Vanderbilt to be "published." I personally joined on as one of the founding editorial reviewers probably with hopes as high as Warren and Noah. I thought the journal was a great idea, and it stands as one of the key elements in founding this journal, but unfortunately it has not prospered in the biological sciences the way we had hoped (1 paper in the four issues to date). For one, the name is somewhat restrictive: if you're not an undergrad, why would you want to send a paper out to a journal with the "Undergraduate" distinction in the name? Secondly, as a graduate student, one is not going to essentially waste his/her hard work that could eventually be published elsewhere by placing it in an in-house journal. Third, submitting any paper involves work…unless it's already written and going to waste! While I admire the *VURJ* and its loyal team (of which I am still enthusiastically a part), *Vanderbilt Reviews Neuroscience* has one huge advantage that they do not have, the third great inspiration: 5-page reviews are already required by the program as part of the doctoral candidate qualifying process. Furthermore, peer review is done by the faculty and the graduate student reviews must be deemed exceptional to even pass the first phase of the qualifying exam. But after all the blood, sweat and tears spilled in the creation of these fine documents, they are allowed to waste. This journal puts an end to that by compiling them all, as a single qualifying class, into one volume of which the students and the Program may be proud. The fourth and final inspiration for this journal is its utility as a recruiting tool—for both students *and* faculty. It is our hope that this journal will be our voice in the competitive world of scholarly work, a novel mechanism by which Vanderbilt makes its mark even bigger. Many, including myself, like to brag that we are *not* the "Harvard of the South," they are the "Vanderbilt of the North." To conclude a paragraph that is entirely too long, I hope the graduate students featured in this, the first of what will hopefully be an annual volume, are proud of their work, and eager to continue contributing to our neuroscience community.

One final comment to get the reader pumped-up: naming the journal *Vanderbilt Reviews Neuroscience* may seem not only obvious, but hypocritical given the criticism of the *VURJ* for being too restrictive. This may be true, but the VBI is laying claim to the "*Vanderbilt Reviews*" part of the name. If it's successful, maybe we'll let the Pharmacology Department use the title when they try to catch up to us!

What's next for Vanderbilt Neuroscience? A lot can happen in a year…

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Vanderbilt Reviews Neuroscience (*VRN*) is an open-access journal (website under construction; please see the Public Knowledge Project: http://pkp.sfu.ca/). *VRN* is the official journal of the Vanderbilt University Neuroscience Graduate Program and the Vanderbilt Brain Institute. *VRN* is a collection of reviews submitted by Vanderbilt University Neuroscience Graduate Program students whilst qualifying for doctoral candidacy. The journal also offers highlights and commentary on work being done at Vanderbilt and in Neuroscience laboratories around the world. *VRN* was founded in May 2009 in an effort to consolidate and recognize the hard work done by each class of Ph.D. qualifiers, and is published yearly by the Institute, one volume per year.

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*.

What to Expect

Inside this, the first issue of *VRN*, the qualifying class of 2008 runs the gamut from molecular to behavioral neuroscience, from our understanding of decision-making processes to what we know about human neurological disease and/or disorder.

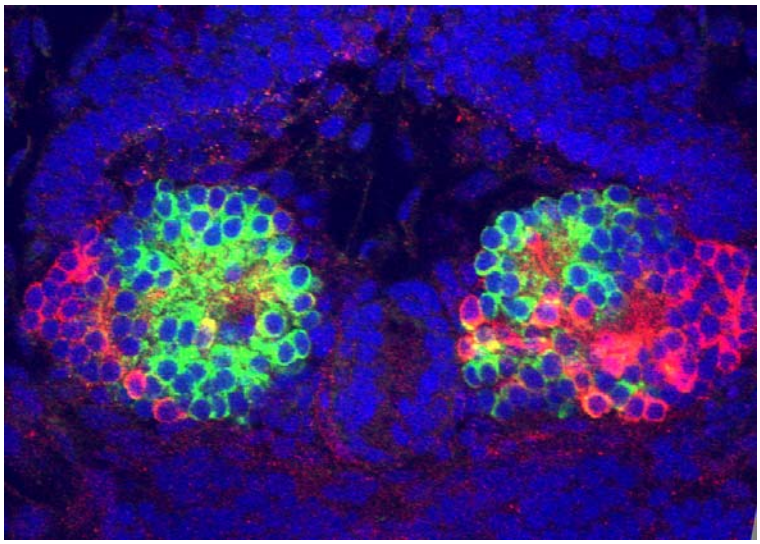
Several great papers have been published in high impact journals this past year, and are highlighted in the "Research Highlights" section of the journal. Buckholtz *et al*. identifies neural circuitry responsible for third-party decision-making (p. 5); Reed *et al*. reported that somatosensory cortex likely serves to integrate information from the hand at multiple levels previously unidentified (p. 7); my collaborators and I show that circadian behavior in mice is the result of population encoding within the brain's biological clock (p. 6); Binda *et al*. report that syntaxin 1a is involved in amphetamine-induced dopamine efflux through the dopamine transporter (p. 8); Cohen *et al.* showed that neurons in the FEF fire at an decreased rate in order to increase selection time when making a saccade in increasingly complex visual search tasks (p. 8).

Dopamine was hot in Vanderbilt University research. Two studies in particular captured the attention of the general public in the past year and are discussed in the "In the News" section of the journal (p. 6), with further elaboration as Research Highlights: Zald *et al*. demonstrated a relationship between D_2-like receptor availability in the midbrain and novelty-seeking behavior (p. 7) and Mazei-Robison *et al*. characterized the A559V mutation in the human dopamine transporter (p. 7). The popularity of dopamine carries through to the candidate reviews, with no fewer than five of the reviews mentioning the neurotransmitter as a major player.

This journal is very much a work-in-progress. The Contents (p. 1) are fairly clear about the reviews inside. How useful they are is up to you. If you have any suggestions, let us know. We hope to do this again.

C. M. Ciarleglio

ON THE COVER…



A single plane confocal image of the zebrafish habenulae at 2 days post-fertilization. The paired habenulae, components of the dorsal diencephalon, asymmetrically express proteins in wild type embryos. In this case, the *big time* mutant displays more symmetrical expression of Leftover (green), though Right on protein distribution (red) appears to be unaffected (To-Pro nuclear dye in blue).

-Caleb Doll

From whence comes judgment

"…the idea that humans may be hard-wired for retributive punishment… may not necessarily be the best or most just."

Every day, juries in courtrooms around the world are charged with the tasks of assessing a defendant's guilt and recommending appropriate punishment. Despite the ubiquitous nature of these processes in human civilization, relatively little is known about the neural mechanisms underlying so-called "third-party punishment." In a recent report in the journal *Neuron*, an interdisciplinary team of researchers at Vanderbilt University investigated the neural circuit activation associated with third-party decision-making.

Buckholtz *et al*. presented human subjects with scenarios in which a fictional character named "John" had Responsibility, Diminished Responsibility, or No Responsibility for a crime which ranged in severity from theft to murder. Subjects' brains were scanned using fMRI while they were presented the scenario and then allowed to decide on an appropriate level of punishment.

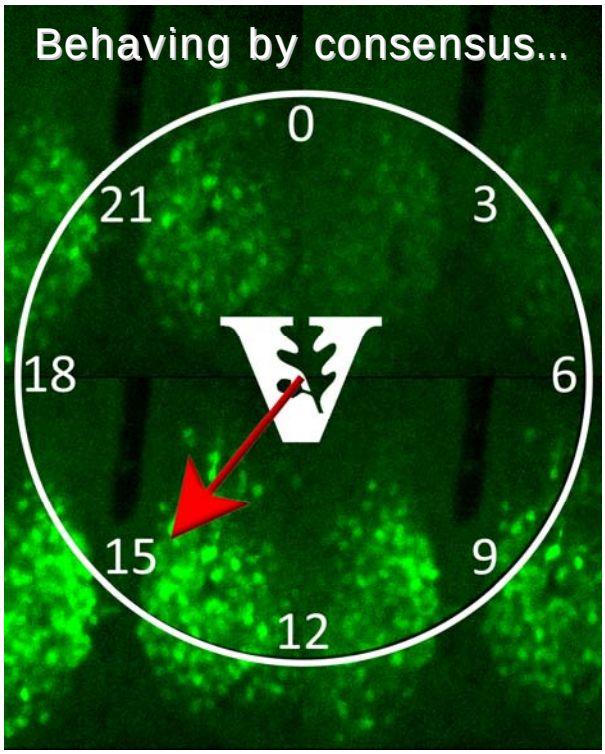
Subjects demonstrated a strong behavioral relationship between their chosen level of punishment and the category of the crime, with the most severe levels of punishment dealt to the most heinous crime scenarios. Additionally, a post-scan questionnaire indicated that subjects exhibited a similar relationship between arousal level and the category of the crime.

fMRI scans indicated that brain-region-specific activation was dependent on "John's" level of criminal responsibility, with the right dorsolateral prefrontal cortex (rDLPFC) and the bilateral anterior intraparietal sulcus (aIPS) being activated more by scenarios in which "John" is Responsible for a crime than when he has either not committed a crime (No Responsibility) or when justifications or excuses mitigate his criminal responsibility (Diminished Responsibility). In contrast, the temporo-parietal junction (TPJ) exhibited greater activation in response to Diminished Responsibility scenarios than Responsibility scenarios. These associations are intriguing given that the rDLPFC is known to be involved in response selection, while the TPJ is known to be involved in processing a person's awareness of other people's mental states, such as their intentions and perspectives.

Interestingly, the authors found that fMRI activation intensity in the rDLPFC did not correlate with the level of punishment assigned by the subject. Instead, the right amygdala, posterior cingulate, temporal pole, dorsomedial and ventromedial prefrontal cortex, and inferior gyrus were found to be involved in determining punishment amount independent of responsibility. Furthermore, this result suggests that assignment of punishment involves a well-classified social and affective neural processing circuit.

Overall, this study demonstrates that third-party punishment is not mediated by a single neural circuit. Rather, multiple circuits and brain regions are recruited to perform processing in legal decision-making—circuits and regions that have been shown to be involved in moral judgments and social norm enforcement behavior (such as the assessment of economic fairness). These findings raise an important issue about legal structure and practice: these data support the idea that humans may be hard-wired for retributive punishment, and may undermine more recent theories that people punish from a consequentialist perspective, suggesting that how people are hard-wired to pursue justice may not necessarily be the best or most just. The high subjectivity of judgment based on an emotional response/circuitry is not conducive of replicable, codified law, which may account for the creation of "precedent" in the legal system. This observation leads one to wonder what influence the establishment of large-scale human cooperation (civilization) had on the evolutionary formation and assignment of neural circuit roles, or vice-a-versa, and what role these processes might have played in the formation of large-scale social and legal norms.

Original Research Article:
JW Buckholtz, **CL Asplund**, PE Dux, DH Zald, JC Gore, OD Jones and R Marois (2008). The Neural Correlates of Third-Party Punishment. *Neuron*. **60**: 930-940.



Circadian rhythms are a nearly ubiquitous feature of life on earth, and are controlled in mammals by the suprachiasmatic nuclei of the hypothalamus (SCN). Since its identification as the primary clock more than 30 years ago, how neurons within the SCN control circadian physiology and behavior has been a mystery. In a study recently published in the *Journal of Neuroscience*, Ciarleglio and colleagues demonstrated how neurons within the SCN worked together as a population to control circadian behavioral rhythms.

Circadian rhythms are controlled in mammalian tissues by a set of clock genes that include some that are expressed robustly during the daytime (e.g. *Period1*). Neurons within the SCN are thought to be synchronized by vasoactive intestinal polypeptide (VIP). In this study, the authors used a dual-transgenic reporter mouse with a short half-life *Period1* promoter-driven green fluorescent protein (*Per1*::GFP) and a knockout for *VIP* to study the relationship between neuronal rhythm synchrony *ex vivo* and robust behavioral rhythmicity *in vivo*. *Per1*::GFP mice wildtype, heterozygous or knockout for *VIP* were behaviorally characterized in a light-dark cycle or in constant darkness, then their brains were extracted and their SCN imaged using time-lapse confocal fluorescent microscopy to observe the expression of GFP *ex vivo*.

Behaviorally, $VIP^{-/-}$ *Per1*::GFP mice were arrhythmic, and more phase advanced than $VIP^{+/-}$ and $VIP^{+/+}$ mice, which were found to exhibit strongly rhythmic behavior with normal behavior onsets in LD and in DD. These results support previous reports that $VIP^{-/-}$ mice had disrupted behavioral rhythms. *Ex vivo* (how the authors refer to acute *in vitro* culture) rhythms were also disrupted in $VIP^{-/-}$ mice, such that they expressed much less neuronal synchrony in the phase of *Per1*::GFP expression than $VIP^{+/-}$ and $VIP^{+/+}$ mice. The authors statistically correlated the degree of neuronal synchrony within an SCN to the power of the same animal's behavioral rhythm, and demonstrated a significant relationship between the two measurements. They found that as the amount of neuronal phase variance increased *ex vivo*, the power of the behavioral circadian rhythm decreased, suggesting that the population of neurons as a whole controlled behavioral output.

The authors also reported two other novel findings. First, the proportion of rhythmic neurons in $VIP^{-/-}$ mice was not statistically different from $VIP^{+/-}$ and $VIP^{+/+}$ mice. This is significant because previous studies had suggested that a lack of VIP led to an overall lack of circadian rhythmicity. Instead, the results of this study suggest that it is neuronal asynchrony that results in behavioral arrhythmicity. Second, an advance in *Per1*::GFP expression correlated to the advance of behavioral onset seen in $VIP^{-/-}$ mice, accounting for this strange phenomenon.

This study is significant in that it demonstrated that neurons within the SCN encode behavior as a population, not unlike the population coding seen in the voluntary motor system where the direction of limb movement is controlled by an average population vector in the motor cortex.

Original Research Article:
CM Ciarleglio, KL Gamble, JC Axley, BR Strauss, **JY Cohen**, CS Colwell and DG McMahon (2008). Population Encoding by Circadian Clock Neurons Organizes Circadian Behavior. *Journal of Neuroscience*. **29** (6): 1670-6.

IN THE NEWS...

Vanderbilt University neuroscience researchers received publicity last year for cutting-edge publications.

Zald *et al*. (2008) was covered internationally for the suggestion that a decrease in D_2-like receptors in the human midbrain were responsible for risk-taking and novelty-seeking behaviors (BBC News; ABC News; ScienceNews.org; see "Getting the Dopamine Rush" on the next page).

Mazei-Robison et al. was also covered extensively for characterizing a mutation in the human dopamine transporter that may lead to attention deficit hyperactivity disorder and that responds to amphetamine in an unusual way (Science Magazine; NPR News; Vanderbilt Reporter; see "DAT Leak: A link to ADHD" on the next page).

IN BRIEF…

Integration of stimuli from across the primate hand

JL Reed, P Pouget, HX Qi, Z Zhou, MR Bernard, **MJ Burish**, J Haitas, AB Bonds and JH Kaas (2008). Widespread spatial integration in primary somatosensory cortex. *PNAS USA*. **105** (29): 10233-10237.

Tactile sensation and discrimination are critical functions of the primate hand, yet the integration of signals from the many sensory neurons in the hand is not well understood. Here, the authors provided evidence for widespread sensory input integration in the brain of the owl monkey, *Aotus trivirgatus*. While small minimal receptive fields in monkey primary somatosensory cortex area 3b are important for stimulus localization, the results in this study indicate that integration in area 3b can also span beyond these small receptive fields. Information is integrated not only within digits, but across the hand in a type of global stimulus processing.

Addiction, extinction and not the α_2-adrenergic receptor

AR Davis, AD Shields, JL Brigman, M Norcross, **ZA McElligott**, A Holmes and DG Winder (2008). Yohimbine impairs extinction of cocaine-conditioned place preference in an α2-adrenergic receptor independent process. *Learning Memory*. **15**: 667-676.

Extinction of learned place preference and drug addiction is poorly understood. In this study, the authors investigated the role of the α_2-adrenergic receptor (α_2-AR) in extinction of cocaine-conditioned place preference (CPP) using the α_2-AR antagonist yohimbine in behavioral and electrophysiological tests. The authors reported that yohimbine impaired cocaine CPP similarly in α_2-AR knockout mice and wildtype mice. Because these effects of yohimbine, a relatively dirty drug, were not seen with a more selective α_2-AR antagonist, atipamezole, and because yohimbine produced an electrophysiological depression of glutamatergic signaling in the bed nucleus of the stria terminalis that was also not seen with atipamezole, the authors suggest that the effects of yohimbine on cocaine CPP are independent of α_2-AR.

Getting the Dopamine Rush

DH Zald, RL Cowan, P Riccardi, RM Baldwin, MS Ansari, R Li, ES Shelby, CE Smith, **M McHugo** and RM Kessler (2008). Midbrain Dopamine Receptor Availability Is Inversely Associated with Novelty-Seeking Traits in Humans. *J. Neurosci*. **28** (53): 14372-14378.

Novelty-seeking behaviors are a great predictor for tendency towards drug abuse in that both novelty-seeking and addiction involve dopamine stimulation of reward centers in the brain. In this study, the authors correlated D_2-like (D_2 and D_3) dopamine autoreceptor availability in the midbrain of human subjects using [^{18}F]fallypride, a specific radiolabeled agonist. Human subjects were given a novelty-seeking questionnaire, and then scanned using positron emission tomography. The authors found an inverse relationship between D_2-like receptor availability in the midbrain of subjects and their tendency towards novelty-seeking behavior, leading the authors to speculate that novelty-seekers may be self-medicating by causing the release of dopamine in response to thrills and novel environments.

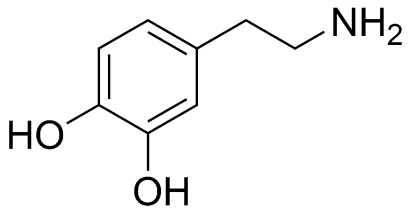
DAT Leak: A link to ADHD?

The dopaminergic system has long been thought to be involved in the etiology of attention-deficit hyperactivity disorder (ADHD). The dopamine transporter (DAT), as a target for ADHD medication, has been characterized for common genetic variants, and yielded several interesting targets for further study. In a paper published recently in the *Journal of Neuroscience* (and later featured as an "Editor's Choice" in *Science*), a team of neuroscientists at Vanderbilt University characterized the human dopamine transporter (hDAT; *SLC6A3*) containing an A559V mutation.

Mazei-Robison *et al*. expressed the hDAT A559V mutation in HEK-293T and found that overall protein expression and cell-surface expression were similar to wildtype hDAT. Using amperometry, the authors found that while levels of dopamine uptake in these cells was comparable to wildtype hDAT, efflux of dopamine was 300% normal. Combining amperometry with whole-cell patch-clamp recording, the authors also found that hDAT A559V exhibited increased sensitivity to intracellular Na^+ which contributed to greater dopamine efflux when depolarized.

Perhaps the most intriguing result from this study was the author's finding that dopamine efflux through hDAT A559V could be blocked by amphetamine (AMPH), which normally enhances dopamine efflux in wildtype hDAT. Because this mutation was originally identified in two male probands with ADHD that were treated with AMPH, this unexpected result suggests a possible mechanism for the efficacy of AMPH as a treatment. Furthermore, the authors found that baseline dopamine efflux in hDAT A559V mimicked the level of efflux seen in AMPH-treated wildtype hDAT. These data strongly suggest that dopamine efflux may be linked to ADHD in a heritable manner, and provide a specific target for further research into therapeutics for the disorder.

Original Research Article:
MS Mazei-Robison, **E Bowton**, M Holy, M Schmudermaier, M Freissmuth, HH Sitte, A Galli and RD Blakely (2008). Anomalous Dopamine Release Associated with a Human Dopamine Transporter Coding Variant. *J. Neurosci*. **28** (28): 7040-7046.



IN BRIEF…

Syntaxin 1a and Amphetamine Fun

F Binda, C Dipace, **E Bowton**, **SD Robertson**, **BJ Lute**, JU Fog, M Zhang, N Sen, RJ Colbran, ME Gnegy, U Gether, JA Javitch, K Erreger and A Galli (2008). Syntaxin 1A Interaction with the Dopamine Transporter Promotes Amphetamine-Induced Dopamine Efflux. *Mol. Pharmacol*. **74** (4): 1101-1108.

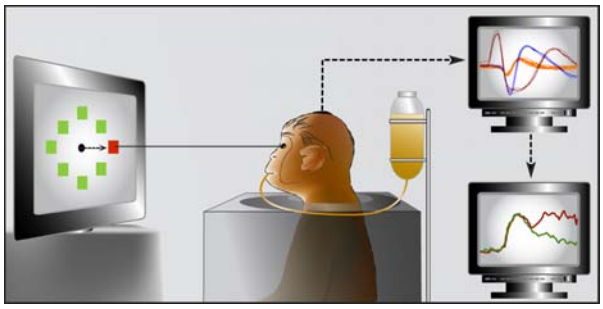
Amphetamine (AMPH) is a psychostimulant with rewarding properties as a drug of abuse. AMPH works on the dopamine transporter (DAT) by causing dopamine efflux through the transporter. In this study, the authors demonstrated that AMPH-induced activation of CaMKII causes syntaxin 1a, a protein critical in mediating vesicle fusion to the plasma membrane, to bind to the DAT N-terminus with greater affinity and increase AMPH-induced dopamine efflux. These results offer insight into the mechanism by which AMPH works as a drug of abuse, and may suggest the target for treatment of AMPH-related drug addiction.

Increased SERT activity and Autism

HC Prasad, **JA Steiner**, JS Sutcliffe and RD Blakely (2009). Enhanced activity of human serotonin transporter variants associated with autism. *Phil. Trans. R. Soc. B*. **364**: 163-173.

This study elaborates on findings of two previous studies (Prasad *et al*. 2005. *PNAS USA*. **102**: 11545-11550; Sutcliffe et al. 2005. *Am. J. Hum. Genet*. **77**: 265-279) showing highly significant linkage between the human serotonin transporter (hSERT) gene (*SLC6A4*) and autism spectrum disorders (ASD). In this study, the authors demonstrated that three variants in hSERT result in a gain-of-function. This increase in transporter activity may contribute to the developmentally atypical aspects of ASD, and may suggest the mechanism by which serotonin plays a role in autism.

Focus among distraction



One of the most widely studied topics in neuroscience is the cognitive process of attention, the process of focusing on one feature of the environment while ignoring all else. Progressing a long way since the early years of relying on introspection as primary methodology, today scientists are investigating the mechanisms that underlie the neural basis of attention. Fundamental to theories of visual attention is the phenomenon that increasing the number of distractors in the environment increases the length of time it takes to select a target due to capacity limitations within the visual system. In their recent report in the *Journal of Neurophysiology*, Cohen and colleagues have investigated the neural basis of this observation.

Macaque monkeys were given the visual task of making a saccade to a target (**T** or **L**) in an array of distractors (**L** or **T**). The task was made more difficult by increasing the number of distractors (1, 3, or 7) in the visual array. Electrophysiological recordings were taken from microelectrodes in the frontal eye field (FEF), a key structure in the visual search network. Both the reaction time, the amount of time it takes a monkey to saccade, and the selection time, the time it takes a neuron to distinguish between target and distractor, were recorded.

The increase in the number of distractors resulted in an increase in the reaction time for both monkeys. The selection time, both across and within individual neurons, was significantly longer with 7 distractors than with 3 or 1. For one of the monkeys, there was also a (across neuron) significant increase in the selection time for 3 distractors vs. 1 distractor. To measure the relationship between the reaction time and the selection time, the investigators fit a linear regression and found that for trials with 7 distractors the regression reached significance. This means that during trials with the most distractors, the selection time of FEF neurons accounts for a significant portion of the variance in the reaction time.

The researchers also found that as the number of distractors increased the peak firing rate of FEF neurons decreased. This decrease in discharge makes it harder to distinguish between target and distractors (signal and noise), possibly accounting for the longer selection times.

This study demonstrates that with increasingly complex visual search tasks, with an increasing number of distractors, FEF neurons fire at a decreased rate and increased selection time, leading to an increase in the amount of time it takes to complete the task. The authors contribute to the theories of visual attention by suggesting a neural mechanism for the limited capacity of the visual system to attend to all inputs within the visual array.

Original Research Article:
JY Cohen, RP Heitz, GF Woodman and JD Schall (2009). Neural Basis of the Set-Size Effect in Frontal Eye Field: Timing of Attention During Visual Search. *J Neurophysiol*. **101**: 1699-1704.

MET: A link to Autism & GI disorders

Characterizing and understanding autism spectrum disorders (ASD) represent great challenges facing neuroscientists today. Accumulating evidence suggests that alterations in the patterning of specific brain structures and circuitry during development may contribute to ASD. The Met tyrosine kinase receptor is important for cell differentiation and organ development. In the developing CNS, Met is thought to facilitate a number of processes including neuronal migration, axon guidance and dendritic arborization by mediating cellular responses to its endogenous ligand, hepatocyte growth factor (HGF). In a paper recently published in The Journal of Comparative Neurology, Judson and colleagues followed up on previous reports relating autism susceptibility to alterations in Met signaling by characterizing Met expression patterns in the developing mouse brain.

The authors used *in situ* hybridization and immunohistochemistry to localize Met transcript and protein, respectively, within the developing murine forebrain. They showed that Met is primarily expressed in specific cortical projection neurons and in certain limbic system components, and that the protein localizes to axonal projections and is particularly enriched in major axon tracts such as the corpus callosum. In addition to characterizing the spatial expression pattern, the temporal pattern of developmental expression was analyzed by quantitative western blot. They found evidence that Met expression levels are highest in the early postnatal developmental period from P0 to P21. This corresponds to the time of mouse brain development in which neurite outgrowth and synaptogenesis occur. This finding further supports a role for Met in the formation of neural circuitry, possibly by facilitating outgrowth and path-finding in forebrain axons. Using an $Emx1^{cre}$ line and a "floxed" Met allele, the authors analyzed mice with a selective ablation of Met in all cells arising from dorsal pallium, which includes projection neurons of the cerebral cortex, hippocampus and some amygdaloid nuclei. This analysis was useful for determining the source of Met expression in the forebrain and further supported the hypothesis that Met is most highly expressed in the axonal projections of neurons, particularly projection neurons of the cortex and components of limbic circuitry.

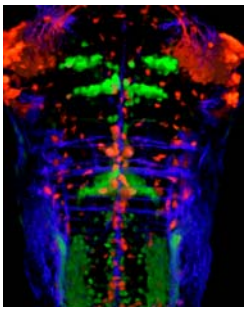
The highest levels of Met expression were observed in the cerebral cortex, and in limbic system associated structures thought to be important for emotional and social function, implicating Met in the establishment and organization of the neural circuitry responsible for maintaining normal emotional and social function. The manifestation of ASD often involves abnormal emotional and social behavior, possibly resulting from a physical disorganization of the circuits involved. This study provides evidence for a potential molecular substrate contributing to developmental abnormalities associated with ASD. Furthermore, it implies a significant role for Met receptor related signaling in normal development of the limbic system and forebrain.

Original Research Article:
MC Judson, **MY Bergman**, DB Campbell, KL Eagleson and P Levitt (2009). Dynamic Gene and Protein Expression Patterns of the Autism-Associated Met Receptor Tyrosine Kinase in the Developing Mouse Forebrain. *J Comp Neurol.* **513**: 511-531.

olig2 and Development

Centuries of painstaking contributions to the human brain atlas have resulted in a nearly gridlocked roadmap of neural networks. Relatively recent genetic characterizations in model organisms have shed new light on the developing brain. These developmental studies hold the capacity not only to decode the origins of neural complexity, but may in turn reveal the molecular nature of neurodegenerative diseases. In a recent paper highlighted on the cover of the *Journal of Neuroscience*, Zannino and colleagues identified neural and glial cell origins in the developing brain, ultimately demonstrating the impact of the *olig2* transcription factor on formation of oligodendrocyte progenitor cells (OPCs) and a specific type of motor neuron (MN) in the zebrafish hindbrain.

Oligodendrocytes are the myelinating cell type of the central nervous system. Through myelination of neural fibers in the CNS, oligodendrocytes contribute to rapid propagation of action potentials. Immature oligodendrocyte progenitor cells are specified from neuroepithelial precursor populations, which also give rise to neuronal cell types. The mechanism of specification and subsequent differentiation from precursor populations has been most intimately studied in the spinal cord, where the neural milieu is relatively restricted as compared to the brain, thereby facilitating the tracing of migratory behavior of cells and



their processes. Previous studies in the Appel laboratory demonstrated that the *olig2* gene is necessary for formation of OPC and spinal motor neurons from the pMN domain of the zebrafish spinal cord. Because oligodendrocytes are present throughout the central nervous system, they extended this hypothesis along the anterior axis to the hindbrain.

Using elegant transgenic strategies and lineage-specific antibody labeling, Zannino *et al*. first characterized neuronal and glial cells in the hindbrain. They witnessed *olig2* mRNA expression specifically in rhombomeres 5 and 6 (r5/r6) of the hindbrain, which was corroborated by enhanced green fluorescent protein (GFP) driven by *olig2* regulatory DNA in transgenic embryos: Tg(*olig2:eGFP*). Antibody staining for Zn8, a marker for somatic abducens motor neurons, was also specific for the 5th and 6th rhombomeres, unlike the broad motor neuron marker, Isl1. Thus hindbrain abducens motor neurons and some OPCs may be specified from a common precursor population. Through time-lapse imaging of Tg(olig2:eGFP) embryos, they next demonstrated that OPCs come from within neuroepithelial precursors in the 5th and 6th rhombomeres of the hindbrain, but that many also arise from *olig2*- precursors elsewhere in the hindbrain.

The investigators next show that the knockdown of the *olig2* gene by targeted antisense morpholino (MO) resulted in a specific effect on hindbrain cells. In morpholino-injected embryos, *olig2* RNA expression was maintained in rhombomeres r5 and r6; however, these cells appeared abnormal at 48 hours post-fertilization, in that most cells appeared to be undifferentiated neuroepithelial precursors and did not possess abducens morphologies. Additionally, BrdU staining for mitotically active cells continued in the hindbrain of MO-injected embryos long after control siblings, suggesting that these cells remain in an undifferentiated state. These results suggest that Olig2 function is necessary for formation of both hindbrain OPCs and somatic abducens motor neurons.

This paper provides several important characterizations of hindbrain cell fate decisions in early development. First, their confocal imaging provides evidence for multiple origins of hindbrain OPCs. In addition, this work shows that timing of *olig2* expression is essential: at early stages the gene is expressed only in the neuroepithelial precursors of rhombomeres r5 and r6, and at later stages only in cells that already possess OPC morphology. Finally, Zannino *et al*. found that *olig2* is also necessary for a class of abducens motor neurons to exit the cell cycle and begin neurogenesis. Ultimately, this work characterizes crucial cell-fate decisions in the developing brain, demonstrating the essential combination of gene regulation and temporal control toward proper specification of both glial and neural cell types in the vertebrate hindbrain.

Original Research Article:
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A note from the Director

In my first year as the Director of the Vanderbilt Brain Institute and the Vanderbilt Neuroscience Graduate Program, I have been continually impressed with the passion for science and dedication to the research endeavor that embodies each of our graduate students. This volume serves as a tangible testament to the exceptional nature of these individuals, and illustrates both the diversity and quality of the neuroscience research enterprise at Vanderbilt. As the first stage in their passage to doctoral candidacy, these reviews serve as springboards to the student's proposed thesis research, and I am delighted to say that each of our candidates demonstrated a strong breadth and depth of knowledge in their chosen research areas while defending these reviews. I am proud to serve in a leadership role for an organization that can join together to highlight its accomplishments in such a novel, impressive and attractive manner, and I am deeply indebted to those (most notably, Chris Ciarleglio) who have taken a leadership role in making this journal a reality.

Yours in science,

Mark T. Wallace, Ph.D.

Gastrointestinal Dysfunction, the MET receptor tyrosine kinase and Autism

Phillip Gorrindo and Pat Levitt*[§]

Phenotypic heterogeneity is a fundamental problem faced by efforts to understand the etiology of Autism Spectrum Disorder (ASD), and likely reflects underlying heterogeneity of genetic and non-genetic susceptibility and causative elements. In addition to the common triad of core impairments, subgroups of individuals with ASD also experience epilepsy, immune irregularities, or gastrointestinal dysfunction (GID). The MET receptor tyrosine kinase has been associated with ASD and is implicated in GI development and repair processes. We hypothesize that pleiotropy of the MET signaling system underlies the co-occurrence of ASD and GID. We seek to leverage the phenotypic heterogeneity of ASD by subsetting populations to enrich underlying genetic signals of risk and improve understanding of ASD etiology. Through this larger goal, we also aspire to develop novel diagnostic tools, interventions and treatments for patients with ASD and GID.

Humans are fundamentally social beings, each embedded in a dense network of social connections. A child with autism, however, never reaches out and remains as a singular node, leaving the surrounding web confused and hurt: we don't know how to interact with a solitary unit, and our natural efforts to connect are doomed to fail. Within the triad of core symptoms of autism (in this document the term autism refers to all autism spectrum disorders)—which include restricted interests and/or repetitive stereotyped behaviors, deficits in language development and communication, and abnormalities of reciprocal social interactions—it is the latter, the social phenotype, that is so difficult for us to comprehend and bear.

Individuals with autism have a number of comorbidities beyond the core triad of symptoms. Epilepsy is seen in one-third of individuals with autism, compared with 2% of a non-autistic population[1]; similarly, autism is often found with mild–to-severe mental retardation. Perhaps most intriguingly, it is anecdotally reported by parents and clinicians who interact regularly with children with autism that there is a high prevalence of gastrointestinal (GI) dysfunction in these children, ranging from chronic constipation and diarrhea to esophageal reflux. Scattered reports in the literature, discussed below, support these claims. Parents of children with autism, who must deal with chronic GI dysfunction in their children in addition to the emotional difficulty of raising a developmentally disabled child, are testaments to the resiliency of the human spirit. This review consolidates the relevant background material for a research project that seeks to understand the nature of these GI comorbidities. With this knowledge, there is the possibility of offering parents and caregivers a new and weighty intervention with two-fold significance: alleviating the GI symptoms can directly influence mood and behavior yielding an altered state that indirectly can increase the potential impact other interventions (behavioral, pharmacological, educational or otherwise) can have on the neurodevelopmental course of the disease.

The link between autism and GI dysfunction has a troubled history. In an original study ten years ago, authored by Wakefield and colleagues[2], twelve children were clinically examined for GI complaints and developmental regression that included the loss of language. Nine of the twelve were said to have autism and all twelve had intestinal abnormalities, including non-specific colitis and ileal-lymphoid-nodular hyperplasia. The authors concluded that these findings were "generally associated in time with possible environmental triggers"—namely, the MMR vaccine in eight of the twelve children. To explain the relationship between these three disparate themes—vaccines, GI dysfunction, and autism—the authors invoked "increased intestinal permeability" (also known as the "leaky gut hypothesis") and the "opioid excess" theory of autism to connect the distant dots. Their reasoning was that the measles component of the MMR vaccine had caused local inflammation in the gut, which altered intestinal permeability, allowing incompletely broken-down peptides to be readily absorbed, which then travel to the CNS where they "may exert central-opioid effects…leading to disruption of normal neuroregulation and brain

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development" and thereby cause autism.

Without ambiguity, the balance of the scientific enterprise has not supported Wakefield's interpretations. In a systematic review of 12 epidemiological studies that have investigated the possible role of the MMR vaccine in the etiology of autism, the overwhelming majority of conclusions do not support a causal link[3]. Although ten of the thirteen original authors have retracted their original interpretation[4], society has already experienced non-trivial consequences. Vaccine rates significantly decreased in the UK[5], and a measles outbreak infected thirty-four people in the US in 2005, primarily among children who were not vaccinated because of parental concerns for adverse events related to the vaccine, such as autism6. Prior to this outbreak, the last outbreak in the US was in 1996, and the disease was declared eliminated in the US in 2000. With this legacy and stigma, any future studies of GI dysfunction in autism must first address Wakefield's story, and then move on.

Despite the Wakefield morass, our group is interested in GI dysfunction in autism. We think that the gut of a child with autism contains important information about the brain of a child with autism. Our reasoning is summarized here and detailed below. We have shown a genetic association between autism and a functional variant in the promoter of *MET*, a receptor tyrosine kinase. Developmental and adult expression patterns of *MET* and related proteins show strong signals in the brain and gut, suggesting pleiotropy. Studies report, with limitations, an increased prevalence of GI dysfunction in children with autism. Finally, we have recently shown a genetic association between co-occurring GI dysfunction in autism and a functional variant of *MET*.

The etiology of autism has a large genetic component. Classic studies of twins showed 92% concordance for autism in monozygotic twins, compared with 10% in dizygotic twins[7,8]. With a population prevalence of one in 150 and a male to female ratio of approximately 5:1[9], the recurrence of autism in siblings (2-8%) is noteworthy[10]. Some of the first genome-wide linkage studies identified chromosomal region 7q, among others, as a site of likely autism vulnerability genes[11,12]—a region which includes the *MET* gene.

Prior to investigating *MET* as a susceptibility gene in autism, our lab had an interest in the role played by MET and its only known ligand, Hepatocyte Growth Factor (HGF), in the development of the cerebral cortex[13,14]. In these studies, we showed that altered MET/HGF signaling in the cortex led to decreased counts of interneurons due to abnormal migration of these cells from the ganglionic eminence during development. Because *MET* is located at 7q31, and because the cerebral cortex shows abnormalities in autism[15], our lab investigated the possibility of MET as an autism vulnerability gene.

In an initial screen using temperature gradient capillary electrophoresis, several SNPs were identified in the coding and non-coding regions of the *MET* gene, which has 21 exons over 125-kb[16]. We investigated the transmission of each SNP using the family-based association test (FBAT), which compares the expected versus observed transmission from parent to affected offspring. Compared to the G allele, the C allele of common variant rs1858830, which is located 20-bp upstream of the *MET* transcriptional start site, was significantly over-transmitted to autism-affected offspring in a combined sample of 743 families ($p < 0.001$). Comparing genotypic frequencies in cases to unrelated controls, the rs1858830 C/C genotype had a relative risk for autism diagnosis of 2.27 (95% CI 1.41 to 3.65), compared to the G/G genotype. It was hypothesized that because of the variant's location in the promoter, that it was functionally important for gene transcription. In transcription assays with a luciferase reporter construct driven by the *MET* human promoter, constructs with the C allele were only half as effective as the constructs with the G allele in driving transcription. Using bioinformatics, it was predicted that the G and C alleles would have different transcription factor binding profiles. Electrophoretic mobility shift assays and subsequent supershift assays comparing the two alleles demonstrated different binding of the transcription factors SP1 and PC4.

In a subsequent study of postmortem brain tissue of individuals with autism compared to matched controls, MET protein levels were found to be decreased two-fold in affected individuals[17]. Moreover, when mRNA levels for other genes involved in the MET signaling pathway were examined, components that activate MET signaling were significantly increased in cases compared to controls. It was proposed that long-term compensatory changes are responsible for this upregulation of mRNAs, suggesting alterations of the entire MET signaling system in autism, rather than only within MET alone. These data combined with the initial genetic findings to provide substantial support for altered MET signaling in autism susceptibility.

Based on the findings of altered mRNAs of MET signaling pathway components described above, a subsequent study investigated genetic association between these components and autism[18]. In this study, the *MET* rs1858830 C allele association found previously was replicated in a new sample of 101 affected families. Additionally, the rs344781 variant T allele in the promoter of *PLAUR* was shown to be

significantly associated with autism in two ways: through over-transmission tested by FBAT ($p < 0.01$), and in frequency tested by case-control analysis ($p < 0.01$). This variant gave a relative risk of 1.93 (95% CI 1.12 to 3.31) for genotype T/T and 2.42 (95% CI 1.38 to 4.25) for genotype C/T compared to genotype C/C. *PLAUR* encodes the receptor for the urokinase plasminogen activator (uPA), which is responsible for cleaving the inactive precursor of HGF into its active form. Using a similar luciferase reporter assay as described above, the T allele induced transcription to a greater degree than the C allele, suggesting functional significance of this variant. Finally, variant rs13238709 C allele in *SERPINE1* was shown to be significantly associated with autism by FBAT transmission studies ($p < 0.05$). Unfortunately, case-control analysis did not support this association. *SERPINE1* encodes the plasminogen activator inhibitor-1, which can suppress cleavage of the inactive precursor of HGF by uPA. This study demonstrates that multiple elements of the MET signaling pathway can confer risk for autism.

The expression patterns of MET and HGF in development and adulthood suggest that MET signaling is pleiotropic—it is not only important in brain development and function, but also in the GI system. An early study found robust expression of both MET and HGF throughout mid and late embryonic development in the mouse[19]. From E10 to E18, both transcripts are present in developing kidney, intestine, lung, liver, pancreas, stomach and muscle. In most cases, MET is found in epithelial tissues and HGF in mesenchymal tissues. Another study looked earlier in development, from E6.5 to E10 in the mouse, and found *MET* and *HGF* expression in the intermediate primitive streak, notochord, and importantly, later neural crest cells[20]. Replicating previous findings, they also showed that later at E13, *MET* and *HGF* are expressed in the developing lungs, liver, and gut. Importantly, similar expression was seen of both *MET* and *HGF* transcript and protein in human fetal tissue, aged 7-24 weeks gestational age, in the developing GI system[21]. Dynamic expression patterns were seen in different tissues—including the esophagus, stomach, small and large intestine, liver, and pancreas—throughout development. Looking in adulthood, another study found similar expression of HGF in human and rat tissues that included the digestive, renal, and reproductive systems[22]. Taken together, these studies demonstrate that MET signaling is also important outside of the brain, in both development and adulthood.

There are scattered reports in the literature that examine the prevalence of GI dysfunction in autistic populations, but many studies are limited in sample size, have inadequate control groups, or lack independent replication. A critical review[23] of these reports concludes that, after failing to find any replicative and rigorous studies, they "found no evidence on which to base a confident statement whether GI symptoms are more common in children with than without autism." Among the five studies they found worth reviewing, prevalence of GI symptoms among individuals with autism ranged from 9% to 84%. It should be noted that, since Wakefield's original study, most studies investigating the intersection of GI dysfunction and autism have intended to address the issue of measles virus causing autism, rather than the significance of GI dysfunction alone. Since that review, one well-executed, prospective study has shown a significantly increased prevalence of GI symptoms in individuals with autism when compared to two matched control groups[24]. The autism group had a prevalence of 70%, compared to a typically developing group with 28% and another group with other developmental delays at 42%.

Another recent study, although with limitations, has interesting findings for the issue of GI dysfunction in autism[25]. The authors report a GI dysfunction prevalence of 23% in a convenience sample of 172 children with autism enrolled in a pharmacology study. The limitations of the study are many: there are no matched controls, there is no indication that this sample is generalizable to the population of individuals with autism at-large, the clinical expertise of a gastroenterologist was not consulted, and their method of assaying GI dysfunction was through either a retrospective medical history review or a short structured questionnaire designed to monitor drug side effects. However, even with those limitations, this is a fascinating paper for what it suggests in the preliminary data it provides. In addition to a medical history, study participants were also asked to complete assessments to characterize their social and cognitive development, as well as levels of anxiety and social withdrawal. Because this GI study was in the context of a larger pharmacology study, responsiveness to treatment was also monitored. The preliminary data in this paper shows that children with autism and GI problems, compared to those without GI problems, have greater levels of anxiety and social withdrawal. Additionally, for children in the risperidone arm of the study, those without GI dysfunction were two times more likely to respond to drug treatment, compared to those with GI problems. These data suggest that GI comorbidities in autism can have important effects on treatment response, and overall disposition, which could ultimately impact the success of other interventions for children with autism.

With these data taken together, our lab tested the reasonable hypothesis that the autism-associated *MET*

promoter variant has an increased association in individuals with co-occurring autism and GI dysfunction[26]. To test this hypothesis we gathered data from an existing research database and gene bank, which yielded 992 individuals in 214 families with a complete medical history and GI condition report. In this sample, 41% of individuals with autism had GI conditions, significantly more than in parents (24%) and unaffected siblings (9%). Although for this analysis the presence of GI condition was scored as a binary outcome (present or not), in this sample the majority of GI conditions in individuals with autism is distributed amongst diarrhea (28%), constipation (33%), and gastroesophageal reflux (5%). When the functional variant in the promoter of *MET* was examined in a subset of 62 families not included in the original study16, the significant association of the C allele with autism was replicated. Additionally, in the 214 family samples, the C allele was significantly associated with the presence of GI conditions. When the 214 family samples was stratified into families with at least one affected child with co-occurring autism and a GI condition, a subsample of 118 families was identified. In these 118 families, the *MET* C allele was significantly associated with co-occurring autism and GI conditions. A potential weakness of this study was that it relied on retrospective, parent-reported GI symptoms. To address this, an additional sample stratification was performed: a subset of 64 families were identified in which at least one child with autism and co-occurring GI symptoms were present, as well as at least one sibling affected with autism but not GI symptoms. The *MET* C allele was significantly over-transmitted to offspring with co-occurring GI symptoms. Because parents were unaware of their offspring's allelic status at rs1858830, this suggests that the association of the *MET* C allele with co-occurring GI conditions and autism is not due to parental reporting bias. This study brings together several themes discussed above to demonstrate that MET signaling is important in a subset of individuals with co-occurring autism and GI conditions, and might reflect a common underlying genetic vulnerability for both central and peripheral pathologies.

Taking all of these data into account, there are several possible biological mechanisms that could explain MET's involvement in GI dysfunction in autism. The development of the enteric nervous system (ENS) could be altered, leading to abnormal function in adulthood. The story of the RET receptor tyrosine kinase in Hirschsprung's disease (HSCR) could be an interesting analogy to MET. HSCR is a congenital disease caused by deficient ENS innervation of the terminal gut. One in 5000 children are affected by this disease and it is usually diagnosed within the first hours after birth, with physical findings of an inability to pass stool, distended abdomen, and vomiting which can lead to tonic contraction of the terminal gut, obstruction, and proximal distention if left untreated[27]. Human mutations in *RET* coding regions are associated with HSCR[28], which are now understood to account for 50% of familial and 15-35% of sporadic cases of the disease. In mice with mutated RET kinase activity, the two primary results are gut aganglionosis and renal agenesis[29]. Conspicuously, the kidney defects are not a common finding in HSCR, suggesting complete ablation of RET activity is an imperfect model of the disease. It has also been recently shown that non-coding mutations in a RET enhancer region are associated with HSCR, suggesting the importance of *RET* dosage in the etiology of the disease[30].

A recent study integrated these facts and demonstrated through a series of *RET* mutants that decreasing *RET* expression to one-third normal levels produces an accurate model of HSCR[31]. These mice lack renal defects, exhibit distal gut aganglionosis, and show incomplete penetrance and a male bias (which are both seen in HSCR[32]). In these mice, neural crest cell (NCC) precursors of ENS cells have altered migration and survival, causing aberrant innervation of the gut. These findings have important advances for the HSCR field: it demonstrates the importance of *RET* dosage and suggests a threshold that could be reached by many different ways; it is a better model of the human disease, addressing obvious inconsistencies in previous models; it demonstrates a combination of migration and survival are responsible for the observed aganglionosis; and finally it clarifies the role of a receptor tyrosine kinase in the development of the ENS.

There is evidence that MET is important in some aspects of NCC development as well. One study showed that transgenic mice which over-express HGF in a variety of tissues characteristically develop ectopic melanocytes[33]. These mice develop melanosis in various parts of the CNS, including the brain, meninges, and spinal cord, as well as hyper-pigmented skin. Interestingly, the authors comment that although abnormal melanocyte development was easily observed, other neural crest derivatives might also be affected in these mice. In passing, they note that intestinal obstruction is conspicuous in these mice and could be related to altered ENS development. Through the studies discussed here, there is a possibly interesting parallel for the story of MET and GI dysfunction. While it might not be the same underlying biology, the story of RET demonstrates how altered NCC and ENS development can impact GI function.

Altered MET signaling could also contribute to GI dysfunction by impacting normal epithelial repair processes in the gut. Recent studies have

demonstrated that HGF can promote epithelial repair in rodent models of GI disorders. One study used a well-characterized model of ulcerative colitis (UC) in rats and showed that exogenous administration of recombinant HGF significantly improved several measures of GI dysfunction[34]. The animals were fed dextran sulfate sodium (DSS) for several days, which is known to induce a phenotype similar to UC, and then continuously administered HGF intraperitoneally. Without HGF, there were decreases in body weight and colon length, and epithelial erosions present—all seen in human UC—associated with DSS administration. HGF administration, in contrast, prevented all of these measures of pathology, suggesting HGF/MET signaling plays an important role in GI epithelial repair in this rodent model of UC. Another study used a rodent model of inflammatory bowel disease with intravenous administration of HGF, and showed decreases in diarrhea and gut inflammation[35]. These studies demonstrate an important possibility for how altered MET signaling could be contributing to GI dysfunction in autism.

Altered ENS development and perturbed epithelial repair are only two of many different possible ways in which MET signaling could be contributing to GI dysfunction in autism. Children with autism can have sensory issues which could lead to behaviorally-mediated GI dysfunction. It is not difficult to imagine a child who has strong tactile aversions refusing to go to the bathroom, leading to chronic GI issues. Additionally, the core feature of restricted interests could drive some children to have poor nutrition, again leading to GI dysfunction. Many of the psychoactive drugs prescribed to children with autism have known GI side-effects[23] which could also contribute to GI dysfunction. For each child, it is possible that any combination of or interaction between these or other possible mechanisms could underlie their GI dysfunction.

We believe the autistic phenotype is deeply heterogeneous, and that by focusing on a clinically significant subgroup of affected individuals, we will boost our genetic signal by focusing on a common underlying biological mechanism. We do not see GI dysfunction as a separate clinical issue for individuals with autism. As investigators, we see it as an opportunity to increase our understanding of the etiology of autism: altered MET signaling, in our hypothesis, affects multiple systems in parallel through a common genetic etiology. As clinicians, we see GI dysfunction as another important element that affects some individuals, making each person unique. With increased understanding of GI dysfunction in autism, we will be able to offer better therapy and interventions to individuals with autism.

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FURTHER INFORMATION

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Examining the Effects of Dopamine System Stimulation During Cortical Axon Guidance

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Dopamine (DA) is a modulatory neurotransmitter that mediates motor function and emotion-based behaviors. Dopaminergic projections throughout the cerebral cortex innervate brain regions implicated in the pathophysiology of neuropsychiatric illnesses such as Parkinson's disease (PD), schizophrenia, and mood disorders. DA is vital to normal brain function and is also involved in sleep, aggression, reward, and appetite. However, the role DA plays during development of the central nervous system has not been fully elucidated. The arrival of DA fibers in the cortex is concurrent with the development of cortical projections and axonal pathfinding of cortical efferents[1]. Recently DA has been shown to affect the migration of interneurons to the cerebral cortex. Animals treated with drugs that increase dopaminergic tone upregulate expression of the axon guidance factor receptors DCC and Unc5c and show neuroanatomical changes in the prefrontal cortex (PFC), a brain region adversely affected in schizophrenia. In addition, DA receptor activation triggers downstream effectors that influence cellular levels of cyclic nucleotides and PKA activity, both of which play a role in growth cone steering and cytoskeletal reformation. Understanding the role of DA receptor activation during development is relevant to the field of psychiatry as schizophrenia is typically first seen in late adolescence and pharmacological treatments for the disorder target D2 DA receptors. This review will examine data that address the role of DA in cortical development, specifically axon guidance. Understanding how DA affects the formation of cortical circuits may shed light on how the DA system functions in diseased brains.

Dopamine
(DA). A modulatory neurotransmitter involved in motor function and emotion. DA also contributes to the establishment of cortical circuitry and brain development.

Frontal Cortex
A region of the brain that plays a role in executive functions, working memory, and attention; the frontal cortex is adversely affected in many psychiatric conditions.

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Dopaminergic neurons originate mainly from two midbrain regions, the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc)[1-3]. SNc neurons project to the dorsal caudate nuclei of the striatum, forming the nigrostriatal pathway[1,2]. The striatum participates in extrapyramidal motor circuits involving the thalamus and motor cortex[4-5]. Altered dopaminergic tone from the SNc to the striatum can result in hypo- or hyper-kinetic movement disorders such as Parkinson's Disease (PD) and Huntington's Disease[4-5]. VTA neurons send dopaminergic projections to the prefrontal cortex (PFC), forming the mesocortical pathway, and to the nucleus accumbens (NAcc), amygdala, and hippocampus to form the mesolimbic pathway[1,6-7]. The mesolimbic system mediates pleasure seeking, reward, and addictive behavior[8]. Decreases in PFC gray matter and reduced PFC activation during cognitive tasks have been seen consistently in schizophrenic patients, making mesocortical dopamine (DA) signaling an area of interest in the field of psychiatry[9-10].

Schizophrenia is a devastating and debilitating mental disorder that affects approximately 1% of the world population[11-13]. The disease is characterized by positive symptoms (hallucinations, psychosis, delusions), negative symptoms (withdrawal, avolition, anhedonia), and cognitive deficits[12]. Weinberger has postulated that schizophrenic patients suffer from an imbalance of DA innervation—an overactive mesolimbic system causes the positive symptoms while an underactive mesocortical system causes negative and cognitive symptoms[7]. Postmortem analysis of schizophrenic brains reveals a decrease in tyrosine hydroxylase (TH)+ and dopamine transporter (DAT)+ axons innervating the PFC[14-15]. The PFC mediates executive function, decision-making, working memory tasks, and critical thinking skills[12]. Individuals with schizophrenia perform poorly on tests that evaluate these skills[10].

DA receptors have long been the target for pharmacological treatment of psychotic disorders[13]. All antipsychotic drugs (APDs) antagonize the D_2 DA receptor, essentially decreasing dopaminergic signaling in patients[13]. APDs relieve positive symptoms of the disease but do little to improve cognitive deficits and negative symptoms[12-13] Overexpression of striatal D_2 receptors in animal models results in decreased DA turnover in the PFC and impaired performance on PFC-mediated working memory tasks[15]. The imbalance of DA circuitry in schizophrenia may underlie PFC dysfunction and involve mechanisms that are not alleviated with current pharmacological therapies. Early life insults, especially those involving the DA system, may profoundly contribute to the pathophysiology of schizophrenia and alter nervous system development

in such a way that it cannot be corrected later in life[11,16]. Understanding how the DA system affects development of the PFC, as well as how DA circuits mature in patients with psychiatric disorders, is crucial to developing treatments for these conditions.

CORTICAL DEVELOPMENT AND DOPAMINE SIGNALING PATHWAYS

During development of the cerebral cortex, neural progenitor cells proliferate in a region bordering the lateral ventricle of the forebrain called the ventricular zone (VZ) [17-18]. Neurons born in the VZ then migrate along radial glial columns to the 6 layers of the cortex in an inside-out fashion, such that deep-layer 6 forms first and more superficial layers form last[12]. Once they have reached their laminar position, neurons extend axonal processes and their growth cones begin the course of axon pathfinding[1-2]. Growth factors and chemical cues present in the neuronal environment guide axons to their targets where synapse formation will occur[19]. An overabundance of synapses is produced during nervous system development and axonal "pruning" occurs in childhood to remove unnecessary synapses[20]. The remaining synaptic connections strengthen, axons become myelinated, and the brain volume increases[20]. The pruning process occurs until late adolescence, commencing with the PFC[7,20]. The overabundance of synapses in the PFC during youth may "mask" the phenotype of schizophrenia until early adulthood, when the first psychotic episode is typically seen and the PFC undergoes reorganization and maturation, resulting in the drastic behavioral changes seen in patients with psychosis[7,11,20]. Postmortem studies in human schizophrenic subjects reveal PFC-specific decreases in neuropil and synaptic protein content, as well as decreased mRNA expression of genes involved in synaptic activity[21-22]. Determining the role DA plays in PFC axon guidance and synapse formation could enhance our understanding of the neuropathological changes seen in psychiatric patients.

DA receptor stimulation has been shown to affect crucial developmental events[17-18,23-25]. Five types of DA receptors exist: D_1 and D_5 are considered "D_1-like" and couple to $G_{\alpha s/\alpha olf}$ to activate adenylyl cyclase, increasing cyclic nucleotide levels; D_2, D_3, and D_4 are "D_2-like" and couple to $G_{\alpha i}$, inhibiting the activation of adenylate cyclase[26-28]. D_1 and D_2 show temporal and spatial differences in their expression patterns[29]. Both have been detected in the frontal cortex and striatum of rodents as early as E12, despite the fact that VTA fibers don't begin to reach the cortex until E16[1,29]. Because second messenger activity of G-protein coupled receptors (GPCR) can influence transcriptional activity, the ratio of D_1:D_2 receptors in a given cell or circuit can have both immediate and long-lasting consequences[30-31]. G-protein mediated second messenger pathways for D_1 and D_2 are differentially affected by cocaine treatment during critical periods of DA system development in animal models[16,27,32]. Drugs of abuse such as amphetamine and cocaine target the DAT and can elicit psychotic symptoms resembling paranoid schizophrenia[12]. These drugs trigger DAT-mediated DA efflux and elevate levels of synaptic DA[33]. Chronic cocaine treatment of pregnant rabbits during critical periods of cortical development (E16-E25) affects dendrite length and D_1 surface density in both the PFC and striatum of offspring[16,27]. D_1-$G_{\alpha s}$ coupling was reduced, while D_2-$G_{\alpha i}$ coupling remained unchanged[16,27]. The surface density of DA receptors and their trafficking patterns following activation is important to study in a developmental context, as they may signal to molecules that regulate the outgrowth and path of PFC axons. Treatment of animals with specific D_1 or D_2 agonists *in utero* and examination of PFC function with cognitive behavioral tasks could reveal an important role of the DA system in the proper assembly of PFC architecture during development.

Cortical circuitry is tightly regulated by a balance of glutamatergic excitation and GABAergic inhibition[23]. The majority of cortical GABA interneurons originate from the ganglionic eminences (GE) of the forebrain and migrate up to the cortex[23]. The GE later develops into the striatum, a region rich in DA receptors[17,23]. Stimulation of DA receptors in forebrain slices from E15 mice affects interneuron migration to the cerebral cortex[23]. D_1 agonists increase migration of neurons from the GE to the cortex, while D_2 agonists have the opposite effect[23]. CDHC, a motor protein that regulates cytoskeleton organization and plays a role in neuron migration, localizes to neurites in D_1 stimulated cultures but is retained in the nucleus of D_2 treated cultures[23,34]. This suggests that the balance of D_1 versus D_2 receptor stimulation is crucial for the formation of inhibitory and excitatory cortical circuitry and that DA receptor signaling might communicate with proteins involved in cytoskeletal reorganization, a key component of axon guidance[23].

Axon guidance
Directional steering of an axon to its target location.

Netrin-1
An axon guidance cue that attracts or repels growth cones.

DCC and **Unc5c**
Receptors for netrin-1; DCC-DCC homodimers signal for attraction towards netrin-1; DCC-Unc5c heterodimers will cause repulsion away from netrin-1.

THE ROLE OF NEUROTRANSMITTERS DURING NETRIN-1 MEDIATED AXON GUIDANCE

Axon guidance factors influence axon pathfinding throughout the entire nervous system[12,19]. Of the four major families of axon guidance cues, netrin-1 and its two receptors, Deleted in Colorectal Cancer (DCC) and Unc5c, play an important role in the pathfinding of cortical efferent axons[35]. Netrin-1 is a secreted guidance cue that is heavily expressed in the area surrounding the striatum. Netrin can cue attraction or repulsion, as well as axon outgrowth[19,36-39]. In the

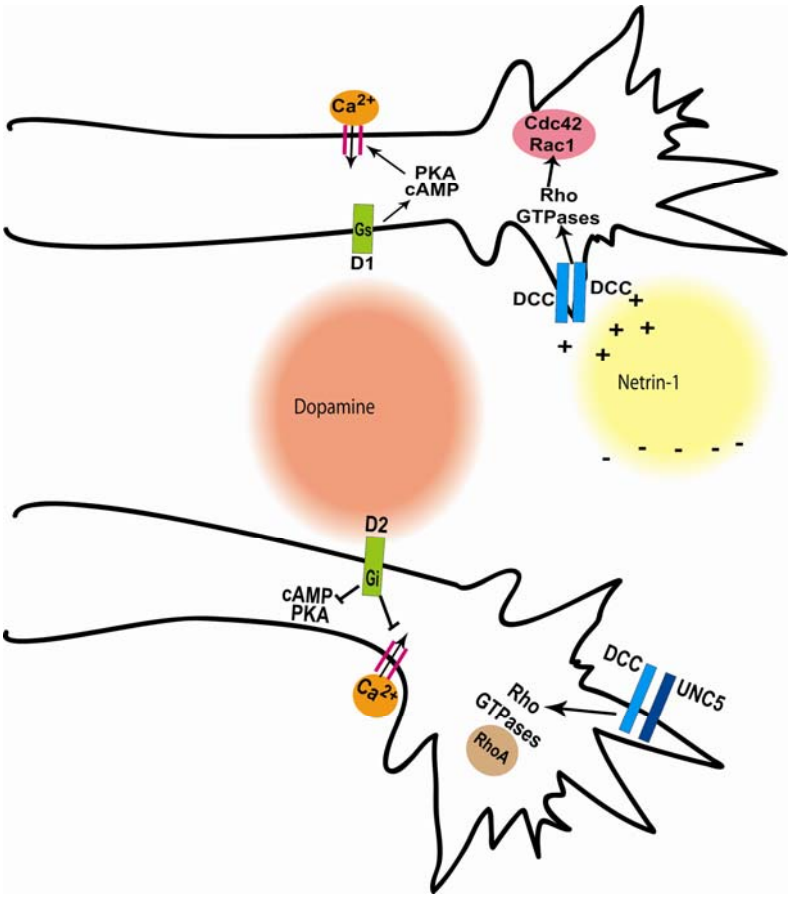

Figure 1 | **Model for DA modulation of ntn-1 mediated axon guidance of cortical efferents.** Frontal cortex cells that express D1 will activate signaling components that can promote insertion of DCC into the membrane and cause attraction toward netrin-1. Conversely, cells containing D2 receptors may promote DCC-UNC5c heterodimers that encode for repulsion away from netrin-1.

presence of netrin-1, DCC homodimers signal for attraction, while Unc5c-DCC heterodimers cause repulsion[19,40-42].

Dopaminergic signaling has also been shown tomodulate expression of netrin receptors[6,43]. Yetnikoff and colleagues showed that amphetamine treatment in adult rodents increased protein expression of both netrin receptors in the PFC as well as the VTA. The fact that adult animals continue to express netrin receptors could be a mechanism of plasticity following the drug treatment, emphasizing the importance of studying netrin-DA system interactions in development as well as adulthood[6]. Conversely, Jassen and colleagues treated neuroepithelial cell lines with D_1 agonists and saw decreased DCC mRNA expression. However, these cell lines only contained D_1 receptors and D_1 agonists increase cyclic nucleotide levels, an event linked with increased DCC activation[40,43]. Evaluating the gene and protein expression of netrin receptors in young animals following drug treatment would be necessary to fully understand how netrin receptors and the DA system affect one another. D_1 vs. D_2 agonists might have opposite effects on netrin receptor expression because they activate different G-proteins and trafficking patterns of the DA receptors[24,28,44-46]. Expression of netrin receptors in the PFC could be important not only for establishing and maintaining DA circuitry in the PFC, but also for maintaining other glutamatergic or GABAergic PFC connections[6].

In addition to DA, another monoamine neurotransmitter, serotonin (5-HT), has been shown to play a role in axon guidance during early brain development[31]. 5-HT receptors are also GPCRs and can affect cyclic nucleotide levels[31]. Stimulation of 5-HT_{1B} and 5-HT_{1D} receptors on thalamocortical axons converts attractive netrin cues to repulsive cues[31]. Both receptors couple to $G_{\alpha i}$ and inhibit the activation of adenylate cyclase[31]. Pharmacological agents that inhibit PKA have the same effect while 5-HT receptor antagonists or drugs that activate adenylate cyclase have the opposite effect[31]. *In vivo* data using *in utero* electroporation of 5-$HT_{1B/1D}$ siRNA in E14 mouse thalamocortical axons also revealed drastic changes in the trajectory of these axons, presumably due to the loss of 5-HT receptor stimulation[31]. This suggests that stimulation of a GPCR-mediated cascade that affects adenylate cyclase production or PKA activation, such as that of DA receptors, can alter the direction of axon growth and implies a role for DA in the pathfinding of axons[40,47]. If the amount of 5-HT receptors present on thalamocortical axons is vital to the development of typical thalamocortical connections, then the abundance and expression of DA receptors in the PFC may be crucial for the development of normal cortical and subcortical connections.

Axon guidance is a cAMP-dependent process and PKA activation triggers biochemical cascades involved in a number of cellular processes related to axon outgrowth and cytoskeleton remodeling[40-41,47]. PKA activation alone does not have the ability to mediate axon outgrowth or guidance but application of netrin and forskolin, a PKA activating drug, enhances axon outgrowth more than netrin alone in commissural neuron cultures[40]. Under basal conditions a small amount of DCC is present on the plasma membrane surface and vesicular stores of DCC are maintained near the growth cone[40]. Binding of netrin to a DCC receptor promotes the recruitment of additional DCC to the cell surface and PKA rapidly enhances the netrin-mediated insertion of DCC into the plasma membrane[19,40]. DCC homodimers are phosphorylated by Src/Fyn kinases that promote the recruitment of a protein complex to the cytoplasmic tail of the receptors[19,41]. Cdc42 and Rac1, members of the Rho family of GTPases, associate with N-WASP to signal changes in actin polymerization,

cytoskeleton reformation, and the formation of lamellipodia and filopodia on the growth cone[41,48]. This results in axon movement and extension of the growth cone towards the source of netrin[19].

As described above, decreases in PKA have been linked to axon repulsion[19,31]. It is not clear how the decrease in cyclic nucleotides affects netrin receptor density and the response of Unc5c to PKA has not been studied in great detail. One hypothesis is that decreases in cyclic nucleotides promote the insertion of Unc5c to the plasma membrane to form heterodimers with DCC, triggering signaling cascades that promote the reorganization of the growth cone cytoskeleton away from the source of netrin[19]. One Unc5 vertebrate homolog, Unc5H2, has been shown to associate with the $G_{\alpha i}$ protein in the presence of cAMP[42]. Under conditions of netrin-mediated attraction, Unc5H2 might bind $G_{\alpha i}$ to ensure attraction and not repulsion[42]. Decreases in cAMP would release Unc5H2 from $G_{\alpha i}$, allowing $G_{\alpha i}$ to inhibit adenylyl cyclase production and decrease cyclic nucleotide levels[42]. Stimulation of GPCRs that contain a $G_{\alpha i}$ protein, such as D_2 and 5-$HT_{1B/1D}$, would therefore promote a decrease in cAMP production and allow free Unc5c to traffic to the plasma membrane to dimerize with DCC[31,42]. The interaction of G-proteins with netrin receptors represents a novel field of study that may explain how neurotransmitter receptors for DA and 5-HT could be affecting axon guidance during development.

CONCLUSIONS

Axon pathfinding represents a fundamental period of nervous system development, as neurons establish synapses to communicate in circuits throughout the brain and the entire body. Evidence suggests that DA receptor stimulation communicates with the netrin family of receptors to contribute to these events. Other axon guidance families including the Ephrins, Semaphorins, and Slits contribute to the patterning of dopaminergic projections[2-3,49-50]. DA receptors could be communicating with their receptors as well. Ephrins have been shown to guide SN neurons to the striatum and the Slit receptor ROBO must interact with DCC to mediate repulsive events[2,4]. A detailed study of the expression and trafficking of netrin receptors following stimulation of dopamine receptors is necessary to address the role of the dopamine system in axon guidance. Mechanisms for axon guidance are different depending on the signal transduction cascade of a given receptor and different types of DA receptors could be important for different families of axon guidance factors. Importantly, expression levels of the membrane bound guidance cues netrin-G1 and netrin-G2 were found to be decreased in post-mortem tissue from patients with schizophrenia and bipolar disorder[51].

The expression of DA and its receptors during early stages of development is necessary for interneuron migration, an event that ensures a balance of excitatory and inhibitory circuitry throughout the cerebral cortex[23]. Activation of DA receptors triggers G-protein mediated cascades that control cAMP production, activation of kinases, and intracellular Ca2+ levels[46,52]. These processes likely communicate with molecules poised to mediate neurite outgrowth, growth cone steering, and cytoskeletal reformation. Stimulation of the DA system in adolescent drug abuse studies reveals lasting neuroanatomical changes that reflect abnormal axon growth in cortical as well as striatal regions[16,27,32]. The functions of the DA system in the PFC and striatum may share some common mechanisms in development. Additionally, some PD patients administered L-DOPA therapy experience psychotic symptoms such as hallucinations while a subset of schizophrenic patients receiving APDs develop extrapyramidal motor side effects[5,13]. Knowledge of early dopamine systems has implications for PD research as the imbalance of excitation and inhibition of motor circuits involving the striatum and motor cortex underlies development of PD.

Understanding vertebrate brain development is crucial for interpreting and developing therapies for complex diseases of the human brain. Further studies must be done to understand how the neurotransmitters that contribute to the pathophysiology of psychiatric illnesses are functioning in embryonic and adolescent brains. The development of a psychiatric patient during childhood and early adolescence may seem fairly normal, but changes in brain chemistry have likely occurred much earlier to elicit such a drastic and enduring phenotype like schizophrenia[11,20]. Other genetic and environmental factors contribute to the disease as well and may adversely affect brain development[11]. Impairment could be permanent and result from alterations made to the cortical circuitry during a critical period of development. In addition, understanding the function of neurotransmitter systems during development has implications for ADHD, which is treated with amphetamines and is commonly seen in young children, as well as autism, a spectrum of developmental disorders in which 5-HT is implicated[31,33]. Further knowledge of the developmental aspects of mental illness could facilitate the correct diagnosis of these disorders at earlier time-points when treatment intervention may be more beneficial, as well as the expansion of pharmacological therapies.

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The Role of the Dopamine Transporter in Attention Deficit Hyperactivity Disorder

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Research focused on neuropsychiatric disorders has shown time and time again that these diseases are extremely complex. As advances in our understanding of disease mechanisms are made, it seems that more questions arise than are answered. This review will introduce the many complexities of attention deficit hyperactivity disorder (ADHD) and focus on the role of the dopamine transporter (DAT) in the disease, specifically addressing various mechanisms of transporter regulation and the primary methods of studying DAT in ADHD.

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ADHD PRIMER

Attention deficit hyperactivity disorder is a relatively common condition characterized by impulsive behavior, hyperactivity, distractibility, and impairments in sustained attention. There are currently no biological markers for ADHD so there is no test for the disorder; diagnosis is based solely on clinical observations and interviews with parents and teachers[1]. The DSM-IV outlines three subtypes of ADHD: predominantly inattentive, predominantly hyperactive and impulsive, and a combined subtype that possesses aspects of both of the other classifications. A positive diagnosis is made when a subject has 6 of 9 inattentive symptoms and/or 6 of 9 hyperactive/impulsive symptoms (**Table 1**)[2]. The DSM-IV-TR diagnostic criteria also require that symptoms are present before age 7, but some would argue that this requirement is too restrictive[2,3].

ADHD is estimated to affect 3-7% of school age children[1] in the general population, a measure in line with the findings of a broad review of more than 100 ADHD surveys that reported a worldwide prevalence rate of 5.29%[4]. However, some researchers contend that ADHD is drastically over- or underestimated on a population level. A recent review of ADHD surveys performed between 1997 and 2007 found ADHD prevalence rates as low as 0.2% and as high as 27%[5]. It is also notable that ADHD exhibits a distinct male-to-female bias, with estimates ranging from 2:1 to as high as 9:1 depending on the subtype[1]. The reasons for this bias are unclear but might include differences in cultural reinforcement of certain gender roles or merely sex differences in biological factors contributing to the disorder itself[2].

Although studies have shown that ADHD symptoms tend to decline as subjects grow older, research suggests that 4% of adults (age 18-44) retain ADHD symptoms[6-9] though some recent studies contend that adult ADHD rates may be as high as 15% for full ADHD diagnosis and as high as 60% for ADHD in partial remission[3,5]. Adults with ADHD have an increased risk for substance abuse[6] and comorbid psychiatric disorders, especially anxiety disorders, eating disorders, anti-social personality disorders, depressive syndromes, tics, and learning (usually reading and spelling) disabilities[10].

Treatment for ADHD typically involves administration of psychostimulants such as methylphenidate (MPH; Ritalin; Novartis Pharmaceuitcals, Basel, Switzerland) or amphetamine (AMPH; Adderall; Shire Pharmaceuticals, Basingstoke, England). Both of these pharmacological agents primarily target the dopamine transporter, but also have limited action on the norepinephrine transporter (NET) and serotonin transporter (SERT)[2]. It has been shown in human studies that MPH blocks DAT in the striatum and effectively elevates extracellular dopamine (DA) concentrations[11]. AMPH functions with a different mechanism—AMPH does block uptake through DAT to a limited degree, but it primarily acts as a DAT substrate, competing with DA and getting transported into the neuron where it reverses the vesicular monoamine transporter (VMAT2), causing DA to leak from vesicles into the cytosol[12]. AMPH also inhibits monoamine oxidase A (MAO-A) to prevent DA from being degraded. In reaction to the AMPH-induced elevation in intracellular DA, DAT reverses its direction of transport and moves DA out of the neuron, thus increasing synaptic DA concentrations and increasing dopaminergic signaling[12]. The efficacy of pharmacological treatments that target the dopamine system immediately implicate dopaminergic signaling as a major player in ADHD symptoms and suggest that dopaminergic dysfunction may underlie ADHD pathology.

Table 1 | **DSM-IV symptom criteria for ADHD.**

Inattention (6 or more)	Hyperactivity/Impulsivity (6 or more)
Fails to attend to details	Blurts out answers
Has difficulty sustaining attention	Difficulty awaiting turn
Does not seem to listen	Interrupts or intrudes
Fails to finish	Talks excessively
Has Difficulty organizing tasks	Fidgets with hands or feel
Avoids sustained effort	Leaves seat in classroom
Loses things	Runs about or climbs
Is distracted by extraneous stimuli	Difficulty laying quietly
Is forgetful	Motor Excess

Taken from Mazei-Robison and Blakely, 2006[2]. Used with permission.

DOPAMINE CIRCUITRY

Neurotransmitters are typically categorized as excitatory or inhibitory depending on how the transmitter affects its target neuron. Dopamine, however, can be excitatory or inhibitory depending on the type of DA receptor it binds—excitatory D1-like receptors (D_1 and D_5) or inhibitory D_2-like receptors (D_2, D_3, and D_4)[13]. When D_1-like receptors are activated, adenylate cyclase (the enzyme that generates cyclic AMP (cAMP)) is stimulated, but D_2-like receptor activation results in inhibition of adenylate cyclase. The activation or inhibition of adenylate cyclase and resulting changes in cAMP levels lead to depolarization (D_1-like) or hyperpolarization (D_2-like) of the cell membrane. Clearly, the dual excitatory and inhibitory roles of DA make the system quite complicated, especially when trying to understand how dopaminergic signaling fits into ADHD pathology[14].

There are four major dopaminergic circuits in the brain (reviewed in [14])—nigrostriatal, hypothalamic-tubero infundibular (HTI), mesocortical, and mesolimbic—and the latter two are most closely linked to ADHD. The nigrostriatal system begins in the substantia nigra pars compacta and projects to the striatum. This circuit primarily regulates motor function and it is the loss of these dopaminergic neurons that leads to the development of Parkinson's disease[15-17]. The HTI pathway starts in the arcuate nucleus of the hypothalamus and projects mostly to the pituitary gland. In this case, dopamine operates under the alias "prolactin inhibiting factor (PIF)" and regulates the secretion of prolactin and luteinising hormone[18].

ADHD, however, is linked to dopaminergic dysfunction in fronto-limbic brain areas[19], specifically the prefrontal cortex[20], nucleus accumbens, and striatum[21] (brain areas involved in ADHD reviewed in [14,22]). These areas are parts of the mesocortical and mesolimbic circuits. Both pathways originate in the ventral tegmental area (VTA), a nucleus medial to the substantia nigra and ventral to the red nucleus in the midbrain[23]. Mesocortical projections go to prefrontal and frontal cortical areas where it regulates information processing, attention, working memory, language, and planning[21]. Mesolimbic projections are directed primarily to the nucleus accumbens (NAc) where they are involved in reward processing and addiction[24], psychosis[25], and major depression[26]. The mesolimbic pathway also makes connections with several other brain regions including the hypothalamus, ventral pallidum, and amygdala[23].

In all of the dopamine circuits, dopamine signaling is terminated by the actions of the dopamine transporter. This plasma membrane protein is located in perisynaptic regions[27,28] and works to recover DA from the synaptic cleft then transport it back into the presynaptic neuron where it is re-packaged into synaptic vesicles for re-release[29,30]. Reuptake of neurotransmitter is one of the main mechanisms utilized in the brain to limit signaling and is seen in several neurotransmitter systems including the other biogenic amines, norepinephrine and serotonin. With such an important role in regulating neurotransmission, is it is easy to speculate how transporter dysfunction could contribute to a disease phenotype.

DAT GENE AND PROTEIN BASICS

The human DAT gene was originally cloned in 1992 by screening a cDNA library derived from the substantia nigra[31]. Further work used phage library screening and restriction site mapping to determine that the fifteen exons and fourteen introns[33] of the DAT gene span over 64 kb of chromosome 5[3]. This work also confirmed that the gene codes for a 620-amino acid protein. To date, there are no reports of alternative splicing in the DAT gene. In addition to the protein-coding sequence, the DAT gene contains a 40-base pair repeat (commonly referred to as a variable number tandem repeat (VNTR)) in the 3'-untranslated region of the gene, with individuals

carrying anywhere from three to eleven copies of the repeat sequence[32]. The precise function of the VNTR is unclear, but it has been shown that the 10-repeat VNTR allele is associated with ADHD[34].

The DAT protein was originally predicted to have twelve transmembrane (TM) domains and intracellularly oriented amino and carboxy termini[35], a structure that was ultimately confirmed when a homologous bacterial leucine transporter (LeuT) was crystallized[35]. Early work on DAT focused on uptake kinetics, inhibitor sensitivity, and ion dependence[36-40], finding that one Cl^- and two Na^+ ions are co-transported with each DA molecule. Work using chimeric DAT-NET fusion proteins later uncovered the structural determinants for the observed Na^+ and Cl^- ion dependence of DAT-mediated transport[40], specifically involving the C- and N-terminal regions (DAT and other related transporters reviewed in ref. 41).

DAT REGULATION AND INTERACTING PROTEINS

At the most basic level, DAT function seems relatively simple—it merely recovers dopamine as it diffuses out of the synapse. However, DAT is a highly regulated protein; its function is finely tuned by phosphorylation, ubiquitination, and several interacting proteins. The most frequently studied DAT-regulator is protein kinase C (PKC). Direct activation of PKC by phorbol esters[42-47] or indirect PKC activation via Gαq-coupled G-protein coupled receptor stimulation[45] leads to decreases in DAT activity, primarily by internalization of DAT to intracellular compartments via a clathrin- and dynamin-dependent process[44,45,47-49]. There is evidence, however, that DAT phosphorylation is not required for internalization[45,47]; it seems that phosphorylation regulates reverse transport through DAT. Collaborative work from the Galli, Javitch, and Gnegy labs showed that alanine substitution for five serines in the DAT N-terminal abolished phosphorylation, but did not affect PKC-induced endocytosis[50]. Rather, the loss of phosphorylation inhibited AMPH-induced DA efflux. Conversely, substitution of aspartates for the N-terminal serines (mimicking phosphorylation) rescued AMPH-induced efflux. It has been suggested that PKC *primarily* regulates DAT via internalization, but that DAT phosphorylaton by PKC or other kinases stabilizes at least some DAT in an "efflux-willing" conformation[2,50].

DAT regulation by kinases, however, is not as simple as PKC-induced down-regulation. In fact, DAT is a substrate for several other kinases. Carvelli and coworkers (2002) found that insulin stimulates DAT activity in a phosphatidylinositol 3-kinase (PI3K) dependent manner that causes a redistribution of DAT to the cell surface[51]. Members of the mitogen activated protein kinase (MAPK) family have also been shown to regulate DAT[46,52]; p42 and p44 MAPK inhibitors lead to decreased DAT activity and plasma membrane expression. Last of all, calcium/calmodulin-dependent protein kinase II (CaMKII) has also been shown to facilitate DAT reversal in response to amphetamine[53]. The precise details of how all of these kinase pathways interact and converge on DAT remain unclear and are being actively researched.

Since it was shown the DAT internalization occurred independent of phosphorylation, researchers began looking for other mechanisms to explain DAT trafficking. Work in yeast[54-57] has shown that plasma membrane trafficking of various transport proteins is regulated by ubiquitination, specifically mono-ubiquitination[58]. Since DAT is trafficked independent of phosphorylation and lacks protein sequence motifs that often serve as sorting signals, researchers in the Sorkin lab examined DAT's ubiquitination state using mass spectrometry[59,60]. These studies showed that upon PKC activation with the phorbol ester PMA, DAT is ubiquitinated in both the N- and C-terminal domains, specifically on lysines 19, 27, 35, and 599. There is some redundancy in the ubiquitination signal, as DATs harboring mutations at single lysines (ubiquitin conjugation sites) have normal trafficking, but endocytosis is disrupted when more than one lysine is eliminated. Sorkin's group went on to utilize RNAi methods to identify Nedd4-2 (neural precursor cell expressed, developmentally downregulated 4-2) as DAT's ubiquitin E3 ligase[61]. This raises an obvious question—if DAT endocytosis occurs independent of PKC-mediated phosphorylation, then why does PKC activation still result in DAT endocytosis? It is known that Nedd4-2 is regulated by phosphorylation[62-64], and, although it has not been demonstrated directly, it is reasonable to hypothesize that Nedd4-2 activity is regulated by PKC[61]. Thus, PKC may be increasing Nedd4-2 activity or somehow allowing Nedd4-2 access to ubiquitination sites on the DAT molecule, and it is the ubiquitination that ultimately causes endocytosis.

The structure of DAT lends itself to many protein-protein interactions, as both termini are oriented towards the intracellular compartment. It comes as no surprise, then, that proteins interacting with DAT are responsible for regulating transport function. For example, several of the kinases that regulate DAT have direct protein-protein interactions with the transporter. It has been shown that both PKCβ-II and CaMKII interact with DAT (PKC on the N-terminal[65] and CaMKII on the C-terminal[53]) and facilitate AMPH-induced DA efflux. DAT phosphorylation is also regulated via DAT's direct interaction with protein phosphatase 2A (PP2A); in a

Table 2 | **DAT-interacting proteins.**

N-terminal Interactions	Function	Ref.
DA D2 Receptor	targets DAT to active synapses; anchors DAT into membrane	69
Receptor of Activated C Kinase (RACK1)	localizes DAT to synapses; may serve as a scaffold for signaling complexes	70
Syntaxin 1A	unknown; inhibits transport in GABA transporter (GAT1) system	71
C-terminal Interactions		
Synuclein	clustering DAT in membrane; DA-induced apoptosis	72
Hic-5	focal adhesion adaptor; likely a scaffold for signaling complexes	73
Piccolo	presynaptic scaffold; role in assembling synaptic active zones	74
Protein Interacting with C Kinase (PICK1)	targets DAT to the plasma membrane; tether PKC to DAT	75

role opposing the kinases, PP2A de-phosphorylates DAT and promotes surface expression[66].

Besides the kinases and phosphatase, several other proteins interact with DAT. It is beyond the scope of this review, however, to address the function of them all in detail (interacting proteins are reviewed in ref. 67). The identity of interacting proteins and a brief description of the proposed role of each interaction can be found in **Table 2**. In nearly all cases, the impact of the protein-protein interaction is not fully understood and is still being actively investigated.

STUDYING DAT IN ADHD

Several studies have been able to make significant links of the dopamine transporter to ADHD. Twin studies have suggested that ADHD is highly heritable—approximately 80% of cases have some significant and identifiable genetic component[22,75] (twin, family, and adoption studies in ADHD reviewed in [76]). A plethora of genome-wide linkage studies have been conducted using various cohorts of ADHD subjects that resulted in linkage at several chromosomal locations including 5p12, 10q26, 12q23, 16p13[77,78]; 17p11[79]; 15q and 7p (although failure to replicate linkage at 16p13 and 17p11)[80]; and 6q12 and 5p13[81]. As the resolution of linkage mapping methods improved, studies identified smaller regions linked to ADHD including 4q13.2, 5q33.3, 11q22, and 17p11[82], as well as 2q21.1 and 13q12.11[83] and 2q35, 5q13.1, 6q22-23, 7q21.11, 9q22, 14q12, and 16q24.1[84]. To summarize, chromosome 5 is most frequently linked to ADHD. Interestingly, the specifically linked region at 5p13 is near the DAT gene locus[85]. The overall lack of consistency among linkage studies may be accounted for by several factors including differences in ADHD diagnosis or the identity of ADHD study populations[2]. It is also possible that ADHD is a complex disorder caused by several common polymorphisms in only a few genes. In this case, it is most likely that several variants in a localized pathway or a functionally related set of genes are contributing to the disorder.

Since genome-wide linkage studies yielded only limited data, many groups opted to study ADHD using a candidate gene approach. In such a method, researchers choose genes that are likely involved in the disorder and look for association of specific alleles to that disorder. In ADHD candidate gene studies, the catecholaminergic neurotransmitter systems are the most common candidates examined (ADHD associated genes reviewed in refs. 86 and 87). Studies of smaller populations as well as larger meta-analyses[88,89] have found association of several genes with ADHD including dopamine β-hydroxylase (DBH)[88-90]; dopamine D_2[90,91], D_4[88-90, 92], and D_5 receptors[88-90]; the serotonin transporter (SERT)[88-90, 92] and various serotonin receptors[88, 89, 92]; acetylcholine receptors[88,92]; monoamine oxidases A[92] and B[94]; synaptosomal associated protein of size 25 kDa (SNAP25)[88-90, 92]; and, most importantly, DAT and the DAT 3'-VNTR[85,88-90,92,95]. The linkage data clearly point to a complex genetic basis for ADHD, and the most consistent findings invariably point to DAT.

Perhaps the most direct link of DAT function to ADHD comes from studying the function of rare coding variants of the DAT protein. Several studies have looked for single nucleotide polymorphisms (SNPs) in the dopamine transporter gene and identified only seven low-frequency coding variants – V24M, V55A, R237Q, V382A, A559V, E602G, and R615C[34, 96-100]. However, only the work of Mazei-Robison and coworkers examined subjects diagnosed with strictly ADHD (i.e. without comorbid psychiatric disorders); the A559V variant was identified in two brothers from this population[34]. Later functional

characterization of this mutant transporter revealed a basal DA leak. DA efflux that typically only occurs upon stimulation (i.e. AMPH treatment) is happening without any pharmacological manipulation[101]. The only other DAT variant with a phenotype of interest thus far is V382A, a transporter that does not properly traffic to the plasma membrane and can exist in the plasma membrane in a transport-inactive state[102].

Research on nearly all aspects of DAT regulation and function are still being actively studied. Many open questions remain regarding DAT regulation, trafficking, and involvement in signaling networks, as well as molecular characterization of rare coding variants. It is noteworthy that there are several useful animal models of ADHD (animal models of ADHD reviewed in ref. 14) including the DAT knockout mouse that displays hyperactivity and learning impairments[30,103,104] and a DAT knockdown mouse that displays hyperactive behavior and allows for pharmacological manipulation since some DAT remains[105-107]. These models allow for *in vivo* studies of DAT mutations as well as DAT mutant function in the context of other genetic manipulations.

CONCLUSIONS

It should be abundantly clear that ADHD is an incredibly complex disorder. The etiology is not fully understood, but it is obvious that several genes and proteins are somehow connected in a diffuse web of interactions, regulations, and cross-communications. The dopamine transporter, however, stands out as a key player in ADHD. Research continues to investigate the function and regulation of DAT. Ultimately, a further understanding of DAT is essential for understanding the role of altered dopamine signaling in ADHD and guiding future therapeutic strategies.

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FURTHER INFORMATION

Randy Blakely's Lab: http://www.blakelylab.org

Identifying the Functional Architecture of the Human Ventral Tegmental Area and the Substantia Nigra using High Resolution Magnetic Resonance Imaging

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The ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc) are subcortical areas within the ventral midbrain that are primary synthesizers of the neurotransmitter, dopamine (DA). Dopaminergic neurons from these areas have widespread projections to many subcortical and cortical parts of the brain. However, to date, the functional architecture of these midbrain regions in humans remains unclear. *In vivo* studies in rodents and non-human primates have shown that the dopaminergic neurons in the VTA and SNc are similar in their firing properties and release of DA[1,2,39]. However, these firing patterns are believed to facilitate multiple functional roles associated with reward related learning, motivation, and other goal directed behaviors[1,50,51,60].

Since most of the studies on the VTA and SNc have been done in non-human species, it is difficult to translate this work into humans and accurately characterize the functional role of these two regions in normal human brain function. Non-invasive functional magnetic resonance imaging (fMRI) is one technique used to study human brain function in vivo. In particular, high resolution fMRI performed in ultra high field magnets (7 Tesla) can be especially beneficial in segmenting the anatomical substructure of brain areas in close proximity. This paper reviews the anatomical layout and functional significance of the VTA and SNc and proposes the use of ultra high field high resolution MRI to study the functional architecture of these two midbrain areas.

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ANATOMY OF THE VTA AND SNc

The anatomy of the VTA and SNc is unique and challenging to study because of its location in the brain, cellular profile and complex interconnections. Nonetheless, an overview of the anatomy is essential to localize the source of an MRI signal, optimize the parameters required to image this part of the brain and correlate it with brain function.

The VTA and SNc are located in the ventral portion of the midbrain brain stem area, and they both vary in size and cytoarchitecture. The VTA is approximately 60mm^3 in size[3] and consists of heterogeneous groups of neurons that are part of the A10 dopaminergic system[5,6]. The SNc is approximately 1100mm in size[3-4] and is part of the A9 dopaminergic system[5,6]. A10 DA fibers contained within the ventromedial midbrain, consist of small diameter (15-30µm), non-myelinated axons that ascend in the medial forebrain bundle (MFB)[7,8]. The A9 DA fibers, on the other hand, vary in size (20-40µm) and extend from the medial lemniscus to the lateral border of the cerebral peduncles[9].

The VTA is further subdivided into separate nuclei based on their location and cellular profile[10,15], and the neuronal populations in these subdivisions tend to be mediolaterally arranged[11]. The SN is topographically divided into two subdivisions, the pars compacta and pars reticulata[9]. The compacta cells (SNc) have larger cell bodies, thicker and longer dendrites, more numerous dendritic segments and denser neuromelanin granules compared to the reticulata cells[12,13,14].

The DA neurons in the VTA have widespread reciprocal connections with sub-cortical and cortical areas of the brain, making this region a major site of information integration. It has reciprocal connections with limbic cortices through the mesolimbic pathway, including the nucleus accumbens (NAc), amygdala, cingulate cortex, and the hippocampal complex[15]. It has efferent and afferent associations with the prefrontal cortex, insular cortex, some sensory, motor and association areas (the mesocortical pathway), and with various nuclei of the thalamus and hypothalamus[15,16,17]. It is also reciprocally connected to the dorsal raphe nuclei, locus ceruleus, various brain stem nuclei, the superior colliculus, reticular formation periaqueductal gray, and the spinal cord[15,17,18,19,20]. Mesocortical projections tend to have their origin dorsorostrally in the VTA[21,22], and the mesolimbic projections originate in the ventrocaudal

VTA[15]. Within the midbrain, the VTA receives glutamatergic input from the laterodorsal tegmentum (in the mesopontine brainstem)[23], and cholinergic input from the pedunculopontine tegmentum[24,25].

Within the ventral midbrain, there is a dorsoventral topography of the DA neurons[26,27,28]. In primates, the dorsal tier of neurons forms a medio-lateral continuum of DA cell groups that include the dorsal SNc, the retrorubral area and the VTA[9,12]. The neurons in this tier are calbindin-D_{28k}-positive, contain low levels of mRNA for DA D2 receptor[27], have dendrites that are oriented mediolaterally, and project to the ventral forebrain as the mesolimbic pathway. In contrast, the ventral tier of neurons consists of the ventral SNc (densocellular zone) and the cell columns extending into the pars reticulata. These neurons are calbindin-D_{28k}-negative, have relatively high levels of mRNA for the DA D2 receptor[27], contain dendrites oriented ventrally, and project to the dorsolateral striatal regions as the niagrostriatal pathway. The neurons in the densocellular zone project to both the ventral and sensorimotor-related striatum[28].

The majority of efferent and afferent pathways of the SNc form the niagrostriatal and striatonigral pathways. The SNc receives topographically organized input from different parts of the striatum in an inverse dorsoventral fashion[32]. Sensorimotor areas of the striatum project to the ventrolateral pallidum and ventrolateral SNc cell columns[29,30,31]. Projections from the central striatum terminate more centrally in both the pallidum and ventral densocellular SNc. Ventral striatum projects topographically into the ventral pallidum, VTA and densocellular SNc[32].

Therefore, the VTA and SNc vary in their cellular architecture along the ventral mediolateral span of the midbrain. Projections to and from the VTA and medial SNc seem to be associated with the limbic areas, whereas the lateral and ventral SNc regions are connected to the association and motor related areas. The next section reviews the functional significance of these structural connections.

FUNCTIONAL PROPERTIES OF DOPAMINE NEURONS IN THE VTA AND SNc

Electrophysiological recordings from the VTA/SNc and their projection sites show DA neurons having a characteristic pattern of activity comprising: (i) a hyperpolarized, inactive state; (ii) a slow, irregular, single spike or 'tonic' firing pattern; and (iii) a burst or 'phasic' pattern of activity[33,34]. Interactions between these distinct firing patterns are known to encode behaviorally relevant signals that facilitate reward related learning, motivation, novelty assessment and other goal directed behaviors.

The phasic firing pattern is dependent on afferent input into the neuron and constitutes a rapid, high concentration efflux of DA released into the synaptic space[35]. This burst release of DA is believed to be the functionally relevant signal sent to post synaptic sites to indicate reward and other goal directed behaviors[1,36]. The DA released in this manner may function selectively on DA receptors localized within or around the synapse and therefore affect only a selected number of post synaptic neurons (for example, in the NAc and striatum) [37].

On the other hand, the single-spike or tonic firing pattern is driven by an intrinsic pacemaker potential[38] that results in a slow changing, low tonic concentration of DA released into the extra-synaptic space[37,39]. The overall activity of DA tonic firing is thus more spatially distributed affecting a large pool of post synaptic neurons (for example, in the ventral striatum) and modulating input from other neurons. The phasic and tonic firing patterns were both observed in the VTA[37] as well as the SNc[33,35,38]. The unique behavioral significance of these two firing patterns is reviewed next.

Single unit studies in non-human primates conducted by Schultz and colleagues[1,40,41,42,43] showed that the phasic firing pattern of midbrain DA neurons (primarily in the VTA and SNc) encodes a learning signal that predicts the error in the occurrence of a rewarding or aversive stimulus. These neurons responded robustly to primary food and liquid rewards and conditioned cues predicting the reward[40,41,44,**45**]. However, they seemed to respond much less to aversive stimuli like air puffs, saline drops to the mouth and foot pinches[46,47]. Even though most of the midbrain neurons measured in the reward prediction error studies were DA in nature, the non-DA neurons, in contrast, were shown to respond robustly to aversive stimuli[47]. Human neuroimaging studies using fMRI have corroborated Schultz's evidence, showing that the VTA and ventral striatum are involved in processing positive prediction errors during conditional associative tasks using appetitive or monetary rewards[**48**,49]. However, due to the limited spatial and temporal resolution of the blood oxygen level dependent (BOLD) response, it is not conclusively credible that the activity seen in the VTA is contributed solely by the phasic firing pattern of the DA neurons.

In contrast, the slowly changing and low concentration tonic firing pattern is thought to be involved in setting up a motivational state, providing initial input to other neuronal systems subserving reward seeking or general goal-directed functions[50,51]. Voltammetry studies[52], micro dialysis studies[53,54] and electrophysiological experiments[55] conducted in the NAc (a major projection site of the VTA) indicate that DA release is maximum during the performance of a behavior or the presentation of a stimulus that triggers a behavioral response. This activity in the NAc is

attributed to its role in engaging with other brain structures to influence parameters for motor activity[56,57]. Thus, the NAc in association with the midbrain DA neurons appears to be involved in higher order sensorimotor functions that are important for motivational processes.

Schultz's studies also showed that the DA neurons in the VTA and SNc fired phasically when the animals were exposed to unexpected or novel aspects of the reward (novel magnitude or reinforcing properties of the reward or novel time of reward delivery)[40,44,45]. Human neuroimaging studies using fMRI also found that the SNc/VTA region preferentially responded to stimulus novelty over other forms of stimulus salience like rareness, target response and negative emotional arousal[58]. However, the spatial resolution in these neuroimaging studies was not feasible to differentiate activity between the VTA and the SNc.

While DA is known to be involved in reward processing, it is more specifically involved in directing attention to salient stimuli in order to prepare an appropriate behavioral response[40,42]. Haber and colleagues[32,59,60] suggested that the process from detecting a salient stimulus to preparing an appropriate behavioral response involves a complex chain of events that recruits an ascending spiral feed-forward organization of the niragro-striatal-niagro pathways. It begins with motivation in the dorsal tier DA cells (VTA, SNc and their connections with the NAc shell), proceeds through cognitive processing in the ventral tier DA cells (SNc and its connections with the NAc core and central striatum); and finally shapes motor outcomes though the ventral tier DA cell columns of the SNc and its connections with the dorsolateral striatum.

In summary, the studies reviewed thus far indicate that the midbrain DA neurons are known to be involved in reward related learning when responding to an unpredicted reward or a cue that reliably predicts the reward. Moreover, they respond preferentially to novel aspects of the stimulus compared to other salience properties. While it is difficult to draw a fine distinction between the role of the VTA and SNc DA neurons, these studies suggest that the VTA DA neurons seem to respond to the motivational, novelty and salience aspects of the stimulus. On the other hand, the SNc DA neurons seem to be associated with preparing an appropriate behavioral response to the stimulus. The role of the midbrain DA neurons in human brain function was not well characterized in the above mentioned fMRI studies. Advanced imaging techniques that can accurately localize the MR signal can provide better insight into the fine grained functional architecture of midbrain substructures like the VTA and SN. Understanding how to achieve this step in imaging technology is discussed in the next section.

IMAGING THE VTA AND SNc

The aforementioned studies have attempted to characterize the function of the VTA and SNc in rodents and non-human primates. However, very little work has been done to functionally delineate the midbrain DA neurons in humans. Given DA's role in reward related learning, novelty assessment and motivation, it would be important to understand how DA produced in these midbrain areas are relevant in human brain function.

While DA release can be directly imaged using PET (Positron Emission Tomography), this technique does not provide high spatial resolution required to segment brain regions in close proximity. Anatomical MR imaging affords the visualization of high resolution images of the human brain and was initially developed for diagnostic purposes. With the advent of current high resolution MRI techniques, areas like the brainstem can be segmented, revealing details approaching those seen in histological specimens[61]. Current three dimensional high resolution MR methods can produce images with an isotropic voxel dimension of 700μm in a 7 Tesla magnet (**Figure 1**).

MRI based on the BOLD response is a potential way to study the functional roles of brain stem areas like the VTA and the SNc. Most fMRI studies involving the brain stem to date have been conducted in low field scanners (1.5 Tesla or 3 Tesla) with imaging protocols that have certain limitations on sensitivity and signal to noise ratios (SNR). However, at higher field strengths (7 Tesla or higher), some of these limitations can be ameliorated because of the ability to view smaller voxel dimensions from more localized regions, and because BOLD signals increase with increasing field strength. However, higher field strength imaging has various technical challenges, especially when imaging the midbrain. Some of these challenges include signal artifacts and distortions caused by magnetic susceptibility variations in the brain and the behavior of radio frequency coils at high frequency.

The location of the midbrain (apposing the interpeduncular cistern) is such that the tissue magnetic susceptibility varies within and across the region, thereby distorting the applied magnetic field. This results in signal artifacts like geometric distortions (macroscopic spatial image distortion) and variations in signal intensity (microscopic, due to dephasing of the proton spins). In order to compensate for susceptibility artifacts, techniques like asymmetric spin echo imaging[62], modified single shot echo planar and spiral imaging[63,64,65], multiple linear gradients[66], and higher order gradients[67] have been used. A recent study used the magnetic susceptibility differences (phase changes) across tissues in a human

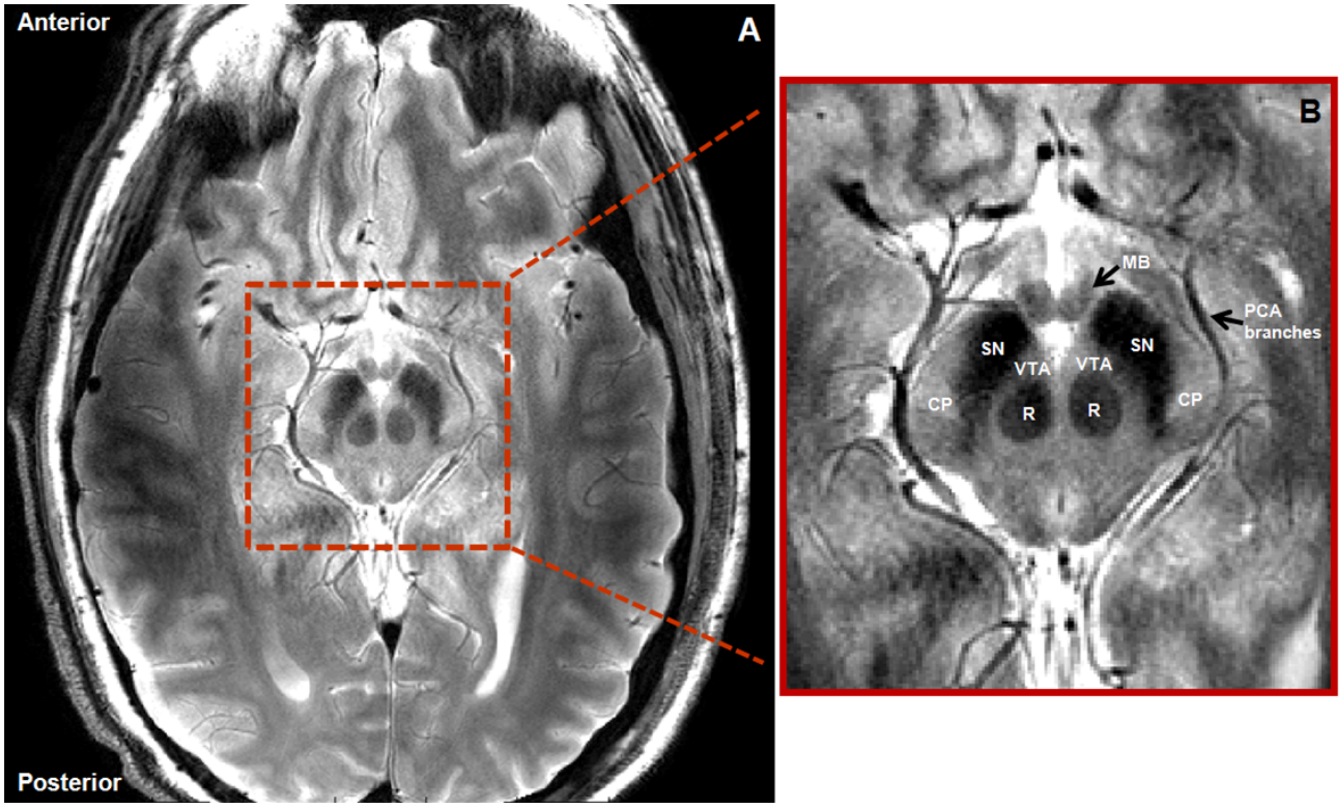

Figure 1 | **7 Tesla MRI image from a T2 weighted GRASE (Gradient And Spin Echo) pulse sequence. a** | Axial image of the midbrain at the level of the superior colliculus; **b** | Enlarged view of the midbrain area highlighted in A. **CP** = Cerebral Peduncles; **SN** = Substantia Nigra; **R** = Red nucleus; **VTA** = Ventral Tegmental Area; **PCA** = Posterior Cerebral Artery; **MB** = Mammillary Body.

7 Tesla to generate high contrast to noise ratios in cortical structures[68]. While none of these techniques can completely eliminate the distortion and signal dropout observed at high field, in various combinations they can be used to improve image quality.

Noise also increases as you go to higher field strengths. The intrinsic noise (thermal noise in the body and from scanner electronics) increases linearly with increasing frequency; while the physiological noise caused by head motion, respiration, and cardiac cycles also increases with increasing field strength. However, specific technical advances are possible to overcome these fields, such as the use of array coils[69] and dynamic shimming[70,76].

While the above mentioned techniques can help to improve image quality, they are not sufficient for accurately localizing fMRI signal (both spatially and temporally) with respect to neuronal activity. The BOLD signal measures neuronal activity indirectly by detecting relative changes in deoxygenated and oxygenated hemoglobin in the vascular region adjacent to the activation site. In order to spatially localize the BOLD signal, different techniques such as diffusion weighted imaging, perfusion weighted imaging (imaging blood flow in capillaries), MR angiography and susceptibility weighted imaging (imaging the venous system at the region of activation) can be used. At higher fields, these methods become more practical because of the higher SNR.

The temporal resolution of fMRI is on the order of seconds, limited mainly by the delay in the hemodynamic correlate of neuronal activity, though in practice, the ability to assess the onset of events is limited by the SNR. Methods to improve the temporal resolution of the data acquisition time include multiple channel acquisition (by using more receiver coils), partial k-space imaging[71] and the development of efficient k-space trajectories that use both x and y gradients to sample k-space in a diagonal or spiral direction[65]. These techniques may only improve the image data acquisition time, but the temporal resolution is still limited by the BOLD dynamics. However, at high resolution, we have the ability to parse out different parts of the BOLD signal as the SNR is higher at higher field strengths.

Apart from reward prediction error tasks, several human fMRI studies conducted in low field magnets have shown BOLD activation in the mesolimbic regions like VTA, SNc, hippocampus and amygdala. These were observed in tasks using pleasant tasting

stimuli[72], monetary rewarding tasks[73], novelty and memory recall tasks[74], reward anticipation and reward related memory recall tasks[75]. However, these studies have not accurately mapped the spatial and temporal profile of the BOLD signal within the midbrain, especially at a resolution required to delineate functional differences between the VTA and SNc.

fMRI techniques mentioned above using high resolution imaging in ultra high filed magnets (like 7 Tesla) promises to improve the localization of the BOLD signal from functionally important areas like the midbrain VTA and SNc. Optimization of already existing imaging techniques and the use of complementary imaging protocols (perfusion weighted imaging, susceptibility weighted imaging, proton density imaging) tailored to the midbrain area would enable us to achieve this.

CONCLUSION AND FUTURE DIRECTIONS

Given what we know of the distinct anatomy and structural connections of the VTA and the SNc, and their functional relevance in various goal directed behaviors, it would be a likely next step to parse out the individual function of these two areas, especially within the human brain. As described in this review, neuroimaging techniques, particularly those using high resolution imaging, offers a plausible approach to investigate the functional architecture of these midbrain regions. This would enable us to ask ecologically valid questions about the role of these midbrain areas in humans, which is typically challenging to do in non-human species. Moreover, the advancement of research methodologies used to examine these midbrain areas can potentially translate into the clinical realm to investigate the role of DA in midbrain related pathologies.

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FURTHER INFORMATION

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Unisensory and Multisensory Disruptions in Autism Spectrum Disorders

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Autism Spectrum Disorders (ASD) are a group of neurodevelopmental disorders which are diagnosed using the following triad of symptoms: impairments in social interaction, impairments in language, and restricted, repetitive, and stereotyped behavior, interests, and activities. A great deal of heterogeneity in the severity of the three symptom classes exists amongst individuals affected by this disorder giving rise to distinct diagnoses such as autism, Asperger's, and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS)[1]. When incorporating all disorders on the autism spectrum, the current prevalence estimate indicates that one in every 150 children is affected by ASD[2]. In addition to the diagnostic triad of symptoms, sensory and perceptual disruptions are frequently associated with ASD. In fact, the original depiction of autism published by Kanner in 1943 included descriptions of sensory abnormalities such as fascination with particular stimuli as well as aversions to innocuous stimuli[3]. Many studies have since been published which seek to characterize these sensory disturbances in ASD. Many researchers have also developed sensory integration therapies which they claim are effective in lessening the severity of symptoms in ASD[4,5,6]. However, many argue that there is no empirical evidence to support the efficacy of these treatments[7,8,9]. This review will highlight studies which examined unisensory and multisensory processing in ASD. Evidence will be described briefly as to why one particular aspect of multisensory integration (i.e. temporal multisensory processing) is likely to be disrupted in ASD. A brief introduction to multisensory processing will follow, and the review will conclude with how the hypothesis of disrupted temporal multisensory processing in autism may be tested.

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SENSORY OBSERVATIONS IN AUTISM

One insight into the sensory disturbances in autism comes from autobiographical reports. For example Temple Grandin, a well known high-functioning professor with autism, describes her hearing experiences as "like having a sound amplifier set on maximum loudness[10]." Other reports indicate difficulty for individuals with ASD to process stimuli from multiple senses concurrently which often results in "sensory overload[11]." Retrospective analysis of home videos of infants who would later be diagnosed as autistic have found symptoms of abnormal reactions to sensory stimuli indicating that sensory disruptions are present even before a diagnosis is made[12].

One strategy for quantifying sensory disturbances in ASD which has been used extensively since 1977 is the sensory questionnaire. These questionnaires are administered to parents or caregivers and usually include items on all modalities and varying reactions to stimulation in each modality (i.e. aversions and fascinations). These studies have shown that abnormal reactions to sensory stimulation as reported by parents are nearly universal in ASD with estimates up to 90%[14,15] of individuals with ASD showing sensory symptoms. These studies have also shown that sensory disruptions are present in multiple modalities and include both hypo- and hyperresponsiveness to stimulation[13-20]. Collectively, this literature represent the entire range of both age and ability in autism indicating that sensory disturbances are an integral component in autism. Although this literature is vital in describing and quantifying abnormal reactions to sensory stimulation, it does not provide any information as to the underlying mechanisms of sensory disruption in ASD.

UNISENSORY PSYCHOPHYSICS IN AUTISM

One method which can be used to examine sensory processing in ASD is psychophysical tasks. Many researchers have tested the ability of both children and adults with ASD to detect and discriminate stimuli from varying modalities. One such study found that high-functioning adults with autism showed enhanced discrimination for highly

similar visual objects[21]. The same group later showed that both children[22] and adults[23] with autism had faster reaction times for detecting visual targets during a visual search task. Similar enhanced perceptual abilities have also been found in the auditory modality. Bonnel *et al* showed that high-functioning individuals with autism were superior in discriminating pitch as well as categorizing "high" vs. "low" tones when compared to controls[24]. O'Riordan *et al* later replicated the finding of enhanced pitch discrimination in autism; however, in the same study they did not find enhanced abilities in the tactile modality for texture discrimination or light touch detection[25]. Recently, Cascio *et al* have found enhanced detection abilities in the tactile modality for some but not all measures. For example, no differences between groups were found for warm/cool detection or ratings of pleasantness for texture. The ASD group did have lower thresholds for thermal pain as well as lower thresholds for vibration detection on the forearm but not the palm[26].

Taken together, the above studies suggest that individuals with autism have superior perceptual abilities; however, other studies indicate that the superior perceptual performance in autism may only be in response to simple stimuli. Bertone *et al* tested this hypothesis by altering the complexity of the stimulus to be discriminated. In this task, participants were asked to discriminate the orientation of a grating which could be luminance-defined (lower order) or texture defined (higher order). Individuals with ASD were superior at identifying orientation for luminance-defined gratings but inferior at identifying orientation for texture-defined gratings indicating that visual stimulus complexity has an inverse relationship with perceptual performance in autism[27]. The relationship between stimulus complexity and perceptual performance in autism was also examined recently by Minshew and Hobson in the tactile domain. In this study the authors differentiated simple vs. complex tactile processing by comparing scores on both simple and complex composite scales between individuals with ASD and without ASD. The simple sensory composite included the following items: localization of cutaneous sensation, sharp vs. dull pressure, and muscle and joint sensation; whereas, the complex sensory composite included the following items: finger-tip writing, tactile finger recognition, wrist shape drawing, and tactile form recognition. Performance was determined by the number of errors made in each composite. Similar to vision, a dichotomy in performance between simple vs. complex processing in ASD was observed. Error rates for the simple sensory composite were similar between groups; whereas, error rates were much higher in individuals with ASD for the complex sensory composite[28]. This study suggests that the inverse relationship between stimulus complexity and perceptual abilities in ASD may be an amodal phenomenon, effecting all modalities. Studies of auditory processing in autism also lend support to this claim. As noted previously, studies examining pitch discrimination in autism tend to show enhanced auditory perception; however, studies utilizing social or verbal auditory stimuli tend to show perceptual impairments[29]. No studies as of yet have directly compared performance on tasks using simple vs. complex auditory stimuli; although, the relationship between stimulus complexity and performance seen in the visual and tactile realms are likely to extend into the auditory realm.

One major theory in autism which explains the dichotomy seen in performance on psychophysical tasks is the theory of weak central coherence which was originally put forth by Frith. This theory proposes that autism is characterized by a processing bias for featural or low-level information at the expense of global processing[30]. One study which tested whether individuals with autism show diminished holistic processing was conducted by Nakahachi *et al*. Participants were asked to detect changes in scenes which could either be related to the theme of the scene or unrelated to the theme of the scene. ASD participants showed lower accuracy for changes related to the theme of the scene but not for changes unrelated to the theme when compared to controls. In the same experiment, participants discriminated between Thatcherized faces and normal faces presented upright or inverted. Typical adults can discriminate Thatcherized faces from normal faces much faster when they are presented upright than when they are presented inverted. This is theorized to occur because people tend to process faces holistically when upright but not when inverted. Participants with ASD showed longer reaction times than controls for upright faces but not for inverted faces. These two experiments together indicate that individuals with autism may have disruptions in processing complex stimuli holistically[31]. Another study found an inverse relationship between disrupted higher order processing (Global Dot Motion Task) and a measure of central coherence (Children's Embedded Figures Test) in ASD also lending support to the weak central coherence model[32].

UNISENSORY EVENT-RELATED POTENTIALS IN AUTISM

Psychophysical measures have proven vital in our understanding of the mechanisms of sensory disruptions in autism; however, much more can be learned about the neural underpinnings of these disruptions by incorporating measures of neural activity such as event-related potentials into studies of sensory processing in ASD. For example, one study

found that the auditory N1c component which is thought to be generated by associative auditory cortex had smaller amplitude and longer latency as well as an unusual lateralization to the right hemisphere. This study indicates that the functioning of the associative auditory cortex may play a role in disrupted auditory processing in autism[33]. Another study examined disruptions in brainstem evoked potentials (EPs) as well as both early and late components of cortical EPs. One participant with autism showed abnormal brainstem EPs; whereas, significant group differences were observed in late components but not early components of the cortical EPs. Because later components are typically more associated with higher order processing than early components, this study indicates that relatively higher order auditory processing in autism may be disrupted[34]. This study may begin to provide a neurological explanation for the dichotomy in perceptual performance discussed earlier in this paper. Samson *et al* addressed this question by reviewing the behavioral and ERP literature on auditory processing in autism. They found that simple stimuli (e.g. pure tones) and simple tasks (e.g. detection) tended to result in superior performance and decreased ERP latencies; whereas, complex stimuli and tasks resulted in inferior performance and ERP activity[35]. Lepisto *et al* also examined whether the different stages of auditory processing may be disrupted differentially. They found evidence of impaired sound encoding as shown by decreased amplitude in response to sound repetition in autism. They also found enhanced discrimination of pitch but disrupted discrimination of duration as evidenced by the mismatch negativity (MMN). They also found disruptions in involuntary orienting to stimuli as shown by the P3a with speech stimuli showing greater disruptions. This study shows that disruptions in auditory processing in autism may occur at multiple levels including involuntary orienting and that they may be more severe for speech stimuli than non-speech stimuli[36]. Other studies also show disruptions in orienting to oddball stimuli in individuals with autism as evidenced by altered MMN or mismatch field (MMF) for auditory[37], visual[38], and somatosensory[38] stimuli.

MULTISENSORY INTEGRATION IN AUTISM

The literature on unisensory processing in autism has given us many clues as to the sensory disruptions in autism. However, much less is known about multisensory integration in autism. The presence of deficient processing in all modalities is suggestive of a larger multisensory defect. A few studies have examined multisensory integration in autism, one of which was published recently by Van der Smagt *et al*. In this study high-functioning adults with autism and controls completed a task which incorporated a well known multisensory illusion known as the flash-beep illusion. This illusion occurs when one flash is presented with two or more beeps, shifting the perception of one flash to two flashes. The authors found no differences between groups on the strength of this illusion, suggesting that multisensory integration of low-level stimuli is intact in high-functioning autism[39]. However, other groups have found evidence of disrupted integration of multisensory verbal stimuli. Williams *et al* presented visual, auditory, and audiovisual syllables such as "ba," "da," and "tha" to children with ASD. The authors found that the children with ASD were less accurate at identifying the unimodal syllables. The children with ASD also did not benefit from the congruent multisensory presentation of "ba" as compared to the incongruent presentation of visual "da" with auditory "ba;" whereas, the controls did benefit from congruent multisensory presentations of "ba." This suggests that the children with ASD were not able to utilize the visual information to improve their performance. However, the deficit in multisensory integration seen in the ASD group could be due to their decreased ability to interpret the visual stimuli. When visual only performance was statistically controlled for, group differences disappeared. Also when a group of children with ASD were trained to lip-read, they did show a benefit from the congruent presentation of "ba" which contrasted with their performance before training[40]. Smith *et al* did find deficits in multisensory integration of speech stimuli in addition to the unisensory deficits. In this task adolescents with autism were presented with auditory speech stimuli in noise and asked to repeat the three key words which they heard. These stimuli were presented in an adaptive staircase procedure in which correct responses resulted in a decrease in speech volume relative to noise whereas incorrect responses resulted in an increase in speech volume relative to noise. This staircase was run twice: once with auditory only stimuli and once with congruent audiovisual stimuli. Both the ASD and TD group showed similar performance on the auditory only task and improvements with the addition of the congruent visual stimuli; however, the TD adolescents showed significantly more improvement from the visual stimuli than the ASD group. Similar to the Williams *et al* study, lipreading was found to be deficient in ASD and significantly affected the ability of the visual stimuli to improve performance. Unlike the Williams *et al* study, this study found that when visual and auditory performance was statistically accounted for, a significant effect of group still remained suggesting disrupted multisensory integration of speech stimuli in autism[41]. The multisensory studies reviewed thus far suggest the

same dichotomy between simple vs. complex/social or verbal stimuli seen in individuals with autism for unisensory stimuli. Mongillo *et al* recently tested this hypothesis by running children with ASD on a battery of multisensory psychophysical tasks which included both tasks incorporating human faces and tasks incorporating inanimate objects. Differences were observed between ASD and TD performance of tasks involving human faces (i.e. male/female face classification, Mcgurk, and AV vowel match/mismatch); however, no differences were observed for tasks involving objects (ball composition and size match/mismatch)[42].

One other aspect of multisensory integration which appears to be disrupted is the distribution of attention within a multisensory object. Lovaas *et al* trained children with autism, mental retardation, and typical development (TD) to respond to a multisensory cue (visual, auditory, and tactile) then tested which of the cues elicited a response. They found that children with TD, and to some extent children with mental retardation, did respond to each stimulus when presented separately. However, children with autism tended to respond to one component of the multisensory stimulus (i.e. visual, auditory, *or* tactile). The authors conclude that this finding may have resulted from an overselectivity of attention within a multisensory object[43]. Studies of event-related potentials during audiovisual selective and divided attention tasks show disrupted attentional modulations of brain responses in autism supporting the claims made by Lovaas *et al*[44,45].

One aspect of multisensory processing which has not yet been studied is the temporal characteristics of multisensory integration. However, there is theoretical evidence that general temporal processing may be disrupted in autism. Brock *et al* theorize that the dissociation between performance on simple vs. complex perceptual tasks might be due to a deficit in temporal synchronization between local networks rather than a general "cognitive style" as proposed by the weak central coherence model[46]. This disruption in temporal binding between cortical and subcortical regions could also manifest as a disruption in multisensory integration as well as a distortion in the temporal characteristics of multisensory binding. One study which examined the perception of temporal synchrony in audiovisual events lends evidence to the assertion that multisensory temporal processing may be disrupted in autism. In this study, children with autism participated in a preferential looking paradigm in which linguistic or non-linguistic stimuli were presented synchronously on one screen and at a delay of 3 seconds on a second screen. Children with TD and children with other developmental disabilities showed preferential looking for both linguistic and non-linguistic asynchronous stimuli; however, children with autism only showed preferential looking for asynchronous non-linguistic stimuli. This study confirms that temporal multisensory processing may be disrupted in autism and that it may also follow the pattern of increased disruptions for complex/social or verbal stimuli than for simple/non-social or non-verbal stimuli[47].

TEMPORAL MULTISENSORY INTEGRATION AND IMPLICATIONS FOR AUTISM

The remainder of this review will be devoted to highlighting the literature on temporal multisensory processing in typical adults and will conclude with future directions for studying whether temporal multisensory processing may be disrupted in autism. The first indications of the temporal properties of multisensory integration arose from studies of multisensory neurons in the superior colliculus. Many of these neurons show superadditive enhancements in response to multisensory stimuli. However, the unisensory components of the multisensory stimulus must be presented in close temporal proximity with one another to produce such enhancements. Interestingly, the unisensory components need not be absolutely synchronous. Instead, a relationship between temporal proximity and enhancements observed exists such that stimuli presented close in time lead to larger enhancements than stimuli present farther apart in time[48]. This same relationship has been observed in numerous psychophysical[49-65] and imaging studies[66-68]. Several studies have also defined a "temporal window" of multisensory integration within which multisensory stimuli are likely to be perceptually "bound"[50-52,69]. One such study, which was published by Shams *et al*, defined a temporal window for the flash-beep illusion introduced previously. In this study, one flash was paired with two beeps with stimulus onset asynchronies ranging from 25 to 250 ms. The second beep could either be presented before or after the flash. The authors were able to use this task to define a temporal window of approximately 100 ms. Future studies could use this task as well as others including the Mcgurk which is also constrained by a temporal window[50] to examine whether autism is characterized by disruptions in temporal multisensory integration. Given the evidence of dichotomies in perceptual performance for simple vs. complex/social or verbal stimuli in autism, it is likely that verbal tasks such as the Mcgurk may exhibit greater disruptions in temporal multisensory processing. However, only further research in this area can confirm this hypothesis.

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FURTHER INFORMATION

Mark Wallace's Lab: http://kc.vanderbilt.edu/multisensory/index.html

The Spinal Neuropeptide Y System is a Potential Target for Chronic Pain Therapeutics

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Millions of people are affected by chronic pain—pain that persists for weeks, months, and even years. Chronic pain costs the economy billions of dollars in health care and disability and the effected individuals lose sleep, money and time from their lives due to their suffering. There are not any successful, long-term treatments for chronic pain, and development of new medications is hindered unfortunately because the precise organization and function of the neural mechanisms underlying nociception are still unclear. For these reasons, it is important to study the nociceptive pathway to learn the identity and function of neurons and transmitters involved, with the goal of identifying new targets for pain therapeutics.

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INTRODUCTION

Many degenerative diseases, nerve disorders, and nerve malfunctions can result in the impairment of physical sensations, such that an affected individual no longer has any sense of being touched, or perceive and ordinary stimulus as painful. These individuals also commonly suffer from chronic pain, which destroys their quality of life. While medicine has many effective ways to treat acute pain, numerous procedures for treating chronic pain have been developed, but have had limited success. Some of these procedures include local electric stimulation, deep brain stimulation, surgeries, alternative medicines like acupuncture, meditation and relaxation techniques and medications. Gaining a better understanding of the signaling molecules and neural networks involved in the pain pathway would be extremely beneficial to creating pain therapies and defining new targets for drug interventions. The goal of this research would be to take advantage of the natural pain transmission pathways and endogenous antinociceptive mechanisms to provide effective pain relief. The dorsal horn of the spinal cord is a prime location for this research. It is a key area of spinal pain transmission[1], but the precise organization and wiring of the neurons is unknown. Several pain-related peptidergic targets have been identified to date in the spinal cord, such as Substance-P and the opioids, and researchers have already taken advantage of these systems to create pain therapeutics. For example, the commonly used analgesic morphine is an agonist of the endogenous mu-opiate receptor[2]. While morphine works well to treat acute pain, the hope is that other neuropeptide systems could be targeted in a similar way to relieve chronic persistent pain. One possible candidate is neuropeptide Y, because recent studies have shown that the spinal neuropeptide Y system is potentially involved in the modulation of nociceptive information[3].

Neuropeptide Y (NPY), a 36 amino acid peptide that is widely distributed throughout the central and peripheral nervous systems[4], has a variety of physiological functions including blood pressure control, feeding, anxiety, and memory[5]. There are at least five different receptor subtypes for NPY (Y1-Y5), with the Y1 and Y2 receptors being the most abundant[6,7]. Acting through its different receptors, neuropeptide Y has been shown to have an excitatory, inhibitory and biphasic effect on cells[8,9]. While more research is needed to confirm if the neuropeptide Y system could be a potential target for chronic pain therapies, the link between neuropeptide Y and nociception has been confirmed by anatomical, behavioral, and pharmacological studies. This review will examine the results from these studies and discuss the potential of using the spinal neuropeptide Y system as a target when developing therapeutics to treat chronic pain.

THE NEUROPEPTIDE Y MEDIATED SYSTEM IN THE DORSAL HORN

In order for NPY to exert a direct effect on nociception, its receptors would need to be located in key sites of nociception. The major spinal cord region involved in nociceptive modulation is the substantia gelatinosa, or the superficial layers (lamina I-II) of the dorsal horn[1].

Neuropeptide Y Y1 receptors in the dorsal horn

The neuropeptide Y Y1 receptor (Y1R) in the

dorsal horn is located primarily post-synaptically and is generally considered to exert an inhibitory effect[9,12]. Neuropeptide Y acts through a G-protein coupled receptor with $G_{i/o}$ subunits to inactivate adenylate cyclase[7,10]. This has an inhibitory effect as the signaling cascade normally activated by G-proteins is inactive. Additionally, the Y1 receptor can activate G-protein coupled inwardly rectifying potassium channels (GIRK). This hyperpolarizes the cell, resulting in its inhibition. Y1 receptors can also influence intracellular calcium levels by activating L-type Ca^{2+} channels[7,10].

There are at least seven different populations of Y1 receptor-expressing neurons in the dorsal horn and area X of the spinal cord. These neuron populations have been classified into types 1-7, with type 1 and type 2 neurons localized in the superficial dorsal horn. Type 1 neurons are found in lamina I-II and are tightly packed, fusiform shaped cells, with rapidly dividing bipolar processes. Type 2 neurons are larger than type 1 and are found in lamina I. Some were identified to be projection neurons by retrograde labeling with Cholera Toxin-B subunit injected at the 9th thoracic segment[11].

It is likely that the Type 1 cells represent the same population of cells described by Zhang *et al*., as small somatostatin-expressing interneurons[12]. This would indicate that Type 1 cells are excitatory interneurons through the indirect evidence that dorsal horn cells expressing somatostatin have been found to co-express the vesicular glutamate transporter 2 (VGLUT-2)[13], making the excitatory transmitter, glutamate, the primary neurotransmitter of those cells. Since NPY peptide co-localizes with γ-aminobutyric acid (GABA) in lamina II[14], NPY may be acting to reduce pain signals through inhibition of the type 1 excitatory interneurons or by acting directly to inhibit the type 2 projection neurons.

Neuron types 3-7 are found throughout lamina III - X and include: type 3, small neurons in lamina III; type 4, large, multipolar neurons in the area between lamina III and IV; type 5, large, multipolar, projection neurons in lamina V and VI; type 6, large, multipolar, projection neurons around the central canal in lamina X; and type 7, large neurons in lamina VIII. It is unknown under which circumstances these neurons are activated, but it is possible that these populations could be activated in situations of inflammation, or nerve injury, and involved in mechanisms of descending inhibition or transmission of nociceptive information to higher brain centers[11].

Neuropeptide Y Y2 receptors in the dorsal horn

Spinal neuropeptide Y Type 2 receptors (Y2R) are located on cell bodies in the dorsal root ganglion (DRG) and are found presynaptically, on nerve terminals, in the dorsal horn; however the anatomy of the Y2 receptor has only been studied in the mouse to date[15]. Activation of the Y2 receptor in the DRG is generally considered to exert an excitatory effect on the cell, which is increased after nerve injury[15]. Since the Y2R regulates N-type calcium channels[16], it can allow more Ca^{2+} to enter the cell and trigger neurotransmitter release. Conversely, activation of the Y2 receptor in the dorsal horn has a net inhibitory effect, since it reduces Ca^{2+} currents and stops the release of excitatory amino acid neurotransmitters.[8] These processes are not yet completely understood and more research is still needed to clarify the data.

INTRATHECAL NEUROPEPTIDE Y REDUCES NOCIFENSIVE REFLEX BEHAVIORS

Intrathecal (i.t.) administration of NPY has been shown to have an antinociceptive effect in the rat. This was first published by Hua *et al*., who found that NPY dose-dependently increased the latency response latency in the 52°C hotplate test[3]. (Typically the response measured in a hotplate test is paw-withdrawal and an "increased latency" indicates that the rat was slower to respond to the stimulus and is therefore interpreted as having decreased nociception.) This research was confirmed by Taiwo & Taylor who found increased paw-withdrawal latency in response to a radiant heat source, in addition to increased hotplate latency[17]. Additional evidence that NPY could be involved in regulating the spinal transmission of nociception came from intrathecal injections of NPY into anesthetized animals, resulting in a reduced nociceptive flexor reflex[18,19]. These behavioral tests show that i.t. NPY reduces protective reflex responses to acute noxious stimuli, but do not necessarily predict an effect in situations of persistent nocifensive stimulation or chronic pain.

Neuropeptide Y is antinociceptive after peripheral inflammation and nerve injury

A common way to model persistent nociception is to inject inflammogens into the plantar surface of the hindpaw. One such inflammogen is complete Freund's adjuvant (CFA), which causes thermal and mechanical hyper-sensitivity for several days[20]. CFA-induced hyperreflexia can be inhibited by i.t. injection of NPY, as shown by increased paw withdraw latencies in the hotplate test[16]. A model of acute peripheral inflammation is the formalin test, where a dilute formalin solution is injected into the plantar hindpaw surface. This damages the tissue, instantly causing intense behavioral and physiological responses that can be measured in terms of licking and flinching behaviors during the 90-minute test, which consists of two distinct phases separated by a relatively quiescent interphase period[21]. NPY dose-dependently inhibited licking behaviors in Phase I

Hyperreflexia
An increased reflexive response to a noxious stimulus.

Formalin Test
A model of acute peripheral inflammation where formalin is subcutaneously injected into the hind paw, where it damages the tissue, instantly causing intense behavioral and physiological responses that can be measured in terms of licking and flinching behaviors.

and[22] licking and flinching behaviors during Phases I and II of the formalin test[21,23].

Persistent nociception can also be induced through nerve injury. The spared nerve injury (SNI) model involves unilateral transection of two out of the three terminal branches of the sciatic nerve[23]. The peroneal and tibial nerves are cut, leaving the sural nerve intact. This results in robust mechanical and thermal nocifensive hyperreflexia (an increased response to a noxious stimulus). The behavioral effects of this injury are seen within 24 hours and last for at least six months[24]. Neuropeptide Y, when administered two weeks after SNI surgery, completely inhibited the enhanced nocifensive responses to mechanical, heat and cold stimuli produced by the nerve injury[22]. These studies indicate that intrathecal injection of NPY is effective in reducing nocifensive reflex responses after peripheral inflammation and nerve injury.

Tail flick test
A test to measure thermal sensitivity where a beam of radiant light is focused on the tail until a response is emitted.

Von Frey Test
A test used to measure mechanical sensitivity, involving nylon monofilaments that, when pressed against tissue until they bend, exert a calibrated amount of force.

Neuropathic Pain
Pain arising as a direct consequence of a lesion or disease affecting the somatosensory system.

Spinal neuropeptide Y system changes after inflammation and nerve injury

The behavioral studies described above suggest that there might be a link between neuropeptide Y and inflammatory or neuropathic pain. It has been found that peripheral inflammation leads to increased levels of NPY and Y1R mRNA transcripts in the dorsal horn[25,26]. This indicates that following CFA injection there are more Y1 receptors, and thus more places for NPY to bind. Additionally, after nerve injury there is increased NPY binding in the dorsal horn[27]. These changes to the NPY system suggest increased NPY signaling and therefore increased inhibition of nociceptive signals. The results support a possible role for neuropeptide Y in the modulation of inflammatory or neuropathic pain.

NEUROPEPTIDE Y ANALGESIA IS BLOCKED BY ANTAGONISTS

The antinociception produced by i.t. NPY can be blocked by simultaneously injecting a NPY antagonist. Two days after unilateral hindpaw CFA injection, Taiwo and Taylor intrathecally administered the NPY Y1 receptor antagonist BIBO3304 with or without NPY. BIBO3304 given alone slightly enhanced the CFA-induced thermal hypersensitivity, indicated by a slight decrease in paw-withdraw latency[16]. This presumably was the result of blocking endogenous NPY from binding to the receptors. When BIBO3304 was given concurrently with NPY, the analgesic effect of NPY was completely inhibited. These effects were similar in the SNI experiments where BIBO3304, when administered along with NPY, completely reversed the anti-allodynic effects of NPY. The Y2 antagonist BIIE0246 also was effective in reducing the anti-allodynic effects of NPY when they were administered together[23]. These experiments provide evidence that the antinociceptive effects of intrathecal NPY can positively be attributed to action of the peptide at its spinal receptors.

Intrathecal
The fluid-containing space around the spinal cord, also called the spinal canal.

Response latency
Measure of time elapsed from application of stimulus to response.

NEUROPEPTIDE Y ANTINOCICEPTION IS INHIBITED IN Y1 RECEPTOR KNOCK-OUT MICE

The antagonist studies showed that both the NPY Y1 and Y2 receptors play a role in modulating nociception. Naveilhan *et al*. further investigated the role of the Y1 receptor in nociception using Y1 receptor knockout (Y1R-KO) mice that were developed at the Karolinska Institute using homologous recombination. The Y1R-KO mice demonstrated a marked nocifensive hyperreflexia, compared with wild-type mice. They showed reduced latencies on hotplate temperatures of 50°, 52°, 55°, and 58°C and also in the tail flick test at temperatures tested between 46° and 54°C. Intrathecal NPY, which has an antinociceptive effect in wild-type mice, had no effect in the Y1R-KO mice on the hotplate tests[28]. The Y1R-KO mice also had a much reduced mechanical threshold, which was measured using the Von Frey test[27,29]. They also showed increased behaviors in response to inflammation and nerve injury. They exhibited increased licking and flinching events during Phase 1 of the formalin test and demonstrated increased pain-related behaviors in response to inflammation caused by capsaicin applied to the hindpaw. Additionally, the response of the knock-out mice to nerve injury was tested using a partial sciatic nerve ligation model. The nerve injury caused mechanical hyperreflexia in wild-type mice, which was notably increased in the knock-out mice[27].

These Y1R knock-out mice experiments were confirmed and elaborated upon by Kuphal *et al*., who used knockout mice developed at the University of Lausanne by Thierry Pedrazzini. Using the CFA model of peripheral inflammation, they found that the dose of CFA required to evoke thermal hypersensitivity for one day in wild-type mice, produced a much longer lasting hyperalgesia in the Y1R-KO mice. CFA also produced mechanical hypersensitivity in both wild-type and KO mice, which was reduced by i.t. injection of NPY in the wild-type, but not the KO mice. Next they tested the mice using the SNI model, which causes thermal hypersensitivity. The anti-hyperreflexia effects of i.t. NPY were reduced in the Y1R-KO mice compared to the wild-type[30].

The enhanced nocifensive reflex responses caused by knocking out the Y1 receptor can likely be attributed to the fact that the endogenous NPY had no available receptors to bind, similar to the NPY antagonist studies. Another theory for the hyper-sensitivity observed in knock-out mice is that they

have increased transcript levels of Substance-P and CGRP, but lower levels of the peptides compared to wild-type. This could indicate that they have an increased release of the excitatory peptides, with a rapid transport of the peptides from the cell bodies, leading to increased nociception[28]. The inability of i.t. NPY to cause antinociceptive effects in the knock-out mice strongly suggests that the antinociceptive reflex effects of NPY are modulated primarily through the NPY-Y1 receptors. Of course, null mice lack Y1R everywhere in the nervous system raising the possibility that the behavioral effects observed were due to changes at supraspinal sites, in addition to any spinal changes.

CONCLUSIONS AND FUTURE DIRECTIONS

Neuropeptide Y receptors are located at the major spinal site of nociceptive regulation. While there is debate over the role of the Y2 receptor in nociception, it is clear that neuropeptide Y acting through its spinal Y1 receptor has an antinociceptive effects. Intrathecal NPY reduced reflexive responses to noxious thermal stimuli and was also very effective at reducing nocifensive reflex responses in situations of inflammation and nerve injury, which are widely used as models of chronic pain. That these effects are specifically linked to the injection of NPY is verified by the fact that they can be blocked by simultaneously injecting a NPY antagonist along with the peptide. Furthermore, the evidence given by the Y1R knockout mice, where no NPY analgesia could be produced, supports an important role for the Y1 receptor in nociception.

Neuropeptide Y receptors have potential as a target for chronic pain therapeutics

The data reviewed in this paper provides a strong foundation for the idea that the neuropeptide Y system could be a target for developing therapeutics for chronic pain, however, there is still more research needed to be done before such a statement can be made for sure. A glaring shortcoming of the research that has been done to date is that all of the behavioral tests used only measure protective reflexes. When looking for a treatment for clinical pain, it is important to use tests that measure what is clinically relevant. Tonic clinical pain is generally associated with prolonged input from c-fibers, which can be activated by low rates of heat transfer[31]. Reflexive tests may not be clinically relevant for testing chronic pain. Additionally, since reflexes involve only the spinal cord, and can be observed in decerebrate animals[17,18], they may not provide reliable information as to what the animal is experiencing. Operant behavioral tests may be better suited for chronic pain research because they force the animal to make decisions on how to deal with noxious stimuli. They can use less intense stimuli and involve cerebral processes. The amount of time spent in contact with noxious stimuli can give researchers an idea of what the animal is experiencing[32]. Until NPY is tested in an operant setting, all we know for sure is that it is an effective reflex modulator.

Additionally, we need a more precise way to investigate what is happening at the cellular level in the dorsal horn—which receptors are involved and which cells express them? The answers to these questions are important since potential therapeutics would act on the spinal NPY receptors. The knock-out animals are a good start, but there are two major downfalls to using them. First, the animals develop without the Y1 receptor and second, the animals have no Y1 receptor throughout their entire neuraxis[28,29]. These issues are problematic since much pain modulation occurs at levels of the brainstem and above, not to mention the other functions of NPY that might be affected by the lack of the Y1 receptor. A better model would be a knockout that can be conditionally turned on after development, or to specifically kill the cells in the spinal cord that express the Y1 receptor using new targeted toxin technology.

The potential for neuropeptide Y to be used as a therapeutic agent in treating chronic pain certainly exists and the actions of NPY after inflammation and nerve injury suggest that it is effective as much more than a reflex modulator. Researchers in this area are on the right track and with the right additional experiments we could possibly have a new peptide system for drug companies to target.

Reflexive tests
Measure involuntary movements made in response to a stimulus.

Operant tests
Measure behaviors that require spinal-cerebrospinal integration and specifically require a learned, motivated behavior that terminates exposure to the noxious stimulus.

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FURTHER INFORMATION

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Dopaminergic Signaling in Development: Regulated for Mediating Biological Functions

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Biogenic amine neurotransmitters are implicated in a wide range of behavioral, cognitive, and homeostatic functions in the mature central nervous system (CNS). Biogenic amines include the catecholamines, dopamine, norepinephrine and epinephrine, as well as acetylcholine and serotonin. Each of these transmitter systems has a unique spatial and temporal pattern of onset during CNS development that has been characterized primarily in rodent models. It is important to note that these neuromodulatory systems appear early during embryogenesis, much earlier than the onset of synaptogenesis, suggesting that they also play important roles in brain development and formation of complex neural circuitry. It is therefore not surprising that alterations to these systems, either by pharmacological agents that affect synthesis or binding in the mature system, or developmentally due to toxic insults or genetic modifications, will have important consequences on brain function. In this review, I will focus on the developmental role of dopaminergic signaling although there are obviously important developmental milestones modulated through other transmitter systems. Regulation of dopaminergic signaling during development is important because current evidence suggests that dopamine (DA) exerts influence during specific sensitive periods in embryogenesis to mediate developmental processes including neuronal process extension. Also, dysregulation of dopaminergic neurotransmission in the striatum and cortex appears to contribute to many neurological and psychiatric disorders, including schizophrenia, Parkinson's disease, attention-deficit hyperactivity disorder, and drug addiction[1-7]. Developmental abnormalities in circuit formation and connectivity may contribute to these disorders even though clinical phenotypes usually become apparent only later in life.

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DOPAMINE IN THE ADULT CNS

The developmental functions mediated by dopaminergic signaling are not currently fully appreciated, however, more is known about the influences of DA in the mature brain. Synthesis of DA involves conversion of the amino acid L-tyrosine into L-dopa by the rate-limiting enzyme tyrosine hydroxylase (TH). Subsequent activity of DOPA decarboxylase results in final conversion to DA. DA is widely distributed in the CNS with important projections into the forebrain. The nigrostriatal tract consists of DA neurons with cell bodies located in the substantia nigra (SN) pars compacta and axonal processes terminating in the dorsal striatum. The striatum is a component of the extrapyramidal motor system and plays an essential role in the coordination of locomotor activity. The mesocorticolimbic pathway is another major forebrain dopaminergic projection. This pathway arises in the midbrain ventral tegmental area (VTA) and provides input to the nucleus accumbens, and some limbic regions including limbic cortical regions (medial prefrontal (mPFC) and anterior cingulate cortex (ACC)). These pathways are those important for mediating behaviors associated with motivation, reward (endogenous systems and drug abuse) and reinforcement, as well as cognitive and executive functions including attention[8-9].

ONTOGENY OF DOPAMINERGIC NEURONS

Detection of TH immunoreactivity has proven to be a useful method for identifying DA neurons during development. Although initial studies were conducted in rodents, parallels have been drawn between other models with available data on rabbits, non-human primates and humans. In the rat midbrain, TH is first apparent at Embryonic day (E)12-13 of an approximate 21 day gestation, and by E14 of an approximate 30 day gestation in the rabbit. Midbrain DA neurons are produced between E36 and E43 of a 165 day gestational period in the monkey[10] and appear during the second month of gestation in humans[11]. Thus in all species examined, dopaminergic neurons are detected very early in development, consistent with a morphogenic role of DA. After initial appearance in the midbrain,

dopaminergic axons project rostrally to target regions in the forebrain. DA axons can be detected in the cortex a few days later[12]. Dopaminergic input is thus already present in the cortex while pyramidal neurons are reaching their laminar positions in the more superficial layers, further support for a morphogenic role of DA. Limbic cortical regions, such as the ACC and mPFC, together referred to as the medial frontal cortex (MFC), receive the densest dopaminergic innervation. The density of TH-positive axons in the cortex increases gradually over development then declines postnatally to reach adult levels during puberty. This protracted postnatal increase in DA content occurs over a time period during which a number of developmental milestones occur that may involve transmitter signaling, such as synaptic maturation and obtaining competency on working memory tasks[13].

DOPAMINERGIC SIGNALING: REGULATION AT THE LEVEL OF RECEPTORS

DA interacts with specific receptor proteins on neuronal membranes to modulate the acute responsiveness of the cell to other synaptic inputs. DA also mediates longer-lasting effects through induction of nuclear changes in gene expression and synaptic plasticity[14]. DA receptors are guanine nucleotide binding protein (G-protein) coupled receptors (GPCRs) characterized by an extracellular N-terminus, intracellular C-terminus and seven membrane spanning segments. The receptors interact through the third intracellular loop with specific G-proteins to induce intracellular second messenger signaling cascades including regulation of calcium and potassium channels on the postsynaptic cell[15-16]. There is also auto-regulatory influence of DA through presynaptic receptor activation. Transmitter action is terminated by re-uptake into the presynaptic terminal by a high affinity plasma membrane dopamine transporter (DAT) in the peri-synaptic area and enzymatic degradation by monoamine oxidase (MAO) or catechol-*o*-methyl transferase (COMT).

Receptors sensitive to DA are divided into two classes based on their pharmacological profiles, sequence homology and signal transduction systems. D_1-like receptors, including the D_1 and D_5 receptor subtypes, couple to the stimulatory $G_{\alpha s}$ protein, of which there exists a long and short isoform, to increase activity of adenylate cyclase (AC) to synthesize cyclic adenosine monophosphate (cAMP). D_2-like receptors, including the D_2, D_3, and D_4 receptor subtypes, have antagonistic functions to D_1-like receptors and couple to $G_{\alpha i/o}$ to inhibit synthesis of cAMP[15-17]. Developmentally, receptor transcripts can be detected in the dorsal striatum and cortex by E14 in the rat and by E12 in the mouse[18-19]. In the monkey, DA receptors appear in target regions of DA input by the first quarter of gestation[20-21] and in humans, DA receptor binding sites have been detected by the twelfth week of gestation[22]. Receptor expression increases throughout prenatal and early postnatal development to reach adult levels of expression between Postnatal day (P)14 and P21 in rodents[23-26]. DA receptors are thus also present early in development, still consistent with a role for DA in mediating circuit formation.

Each receptor subtype possesses distinct cellular and/or regional distributions and pharmacological profile. In the dorsal striatum, dopaminergic signaling is mediated primarily through the D_1 receptor (D_1R) and the D_2 receptor (D_2R) which are expressed in greater abundance than the other subtypes and enriched in specific populations of efferent GABAergic medium spiny neurons, although there is evidence of co-localized populations. Neurons projecting from the striatum to the SN pars reticulata are enriched in the D_1R and co-express the peptide substance P. These neurons are involved in the direct extrapyramidal pathway. Neurons projecting to the external globus pallidus, on the other hand, are enriched in the D_2R and co-express enkephalins. These neurons are involved in mediating the indirect pathway. Receptor protein expression is high in the SN pars reticulata and external globus pallidus, but no receptor mRNA has been detected in these regions indicating that the receptors are present on the axonal process associated with the projection neurons from the striatum[15-16].

DOPAMINERGIC SIGNALING: REGULATION AT THE LEVEL OF G-PROTEINS

G-proteins serve as signal transducers between membrane-bound receptors and internal cellular effector systems. G-proteins exist in hetero-trimeric complexes composed of a G_α subunit in association with $G_{\beta\gamma}$ which exists as a functional dimer. Activation of the G-protein complex is controlled by a regulatory cycle involving receptor-activated exchange of GDP for GTP on G_α, dissociation of the trimer, activation of effector molecules, and inactivation through GTPase activity of G_α. The regulatory protein $G_{\alpha s}$ is responsible for stimulatory G-protein signaling in most cell types, however the striatum contains low expression of $G_{\alpha s}$. In the striatum, D_1R signaling is complicated by the presence of another stimulatory G-protein, $G_{\alpha olf}$ which exists in greater abundance than $G_{\alpha s}$[27-28] and is classically known to be important in olfactory signal transduction. More recently, $G_{\alpha olf}$ has been shown to couple to D_1Rs in the striatum to increase activity of AC[29]. $G_{\alpha olf}$ shares more than 80% sequence homology with $G_{\alpha s}$ but may fall under different regulatory controls[30]. In the striatum, $G_{\alpha olf}$ is developmentally regulated; increasing in expression

from P0 to P14 in mice before reaching a plateau after P14[30]. This developmental trend in $G_{\alpha olf}$ expression is reflected in forskolin stimulated cAMP activity in AC assays in which cAMP signaling increases significantly from P0 to P14 and plateaus at ages thereafter. $G_{\alpha olf}$ may be an important mediator in DA signaling through D_1Rs in the striatum as indicated by the loss D_1R-stimulated cAMP production in $G_{\alpha olf}$ knock-out mice[29]. A blunted cocaine or selective D_1 agonist-induced locomotor response has also been observed in these mice indicating a role for this protein in transducing this DA-mediated response[27].

Apart from $G_{\alpha s}/G_{\alpha olf}$ coupling to activate AC in the striatum, DA receptors have also been implicated in intracellular calcium mobilization through coupling to $G_{\alpha q}$ and activation of phospholipase C[31]. In this pathway, inositol triphosphate (IP_3) and diacylglycerol (DAG) second messengers are generated from phosphatidylinositol (PI) metabolism. Liberated IP_3 then binds intracellular receptors to release calcium from intracellular stores. Activation of the PI hydrolysis pathway has been shown to be triggered by specific D_1-like agonists that can be inhibited by co-application of D_1–like antagonists or co-application of D_2–like antagonists. D_2R-like agonists alone, however, are not able to stimulate calcium release. Additionally, it has been observed that D_1–like agonists differentially stimulate PI hydrolysis and /or AC activity to varying degrees indicating that AC-coupled and PI hydrolysis-coupled D_1Rs are distinct molecular and pharmacological entities[32-34]. It has recently been demonstrated that hetero-oligomers containing D_1 and D_2 receptors associate in neurons and co-activation of the receptors rapidly activates the $G_{\alpha q}$ pathway triggering calcium release and activation of calcium dependent molecules such as CaMKIIα[35-37]. The D_1R/D_2R hetero-oligomeric activation of $G_{\alpha q}$ is distinct from D_1R and D_2R activation of $G_{\alpha s}/G_{\alpha olf}$ or $G_{\alpha i/o}$, respectively[37-38]. D_1R-$G_{\alpha q}$ coupling has also been observed in the cortex, amygdala and hippocampus[39].

DEVELOPMENTAL INSULTS ALTER DOPAMINERGIC SIGNALING

As has been thus far noted, dopaminergic signaling is developmentally regulated at the level of dopamine expression, receptor expression and expression of G-proteins. Evidence from our laboratory and others investigating the effects of prenatal cocaine exposure on brain development in rabbits indicates that prenatal cocaine during a sensitive period in development impairs signal transduction through D_1Rs in the striatum and cortex. Impaired D_1R signaling results in permanent abnormalities existing for the life-time of the offspring including aberrant process elongation in the ACC and altered responsiveness of neurons in culture after D_1R activation with selective D_1R-like agonists[40-41]. This model is just one example of how a pharmacological challenge to developing brain circuits results in long-lasting changes in signaling (D_1R signaling in this case) perhaps as a compensatory mechanism to adapt to the *in utero* environment. Pharmacologically, cocaine interacts with the high affinity transporters of DA, norepinephrine and serotonin. Cocaine binds to the transporters and effectively blocks re-uptake of these monoamines into the presynaptic nerve terminal. As a result, the extracellular concentration of neurotransmitters is increased thus prolonging receptor activation. This effect also occurs *in utero* when a fetus is exposed to cocaine prenatally as cocaine readily crosses the placental barrier to inhibit DA uptake.

Prenatal cocaine exposure results in abnormal regulation of dendritic growth of cortical neurons in regions receiving dense DA input, without like changes in cortical regions receiving dense input from other transmitter systems such as visual or somatosensory cortex which both contain high serotonin content[43,46]. Under normal developmental parameters, it has been shown that DA receptor activation produces opposing growth phenotypes dependent upon the receptors activated and the functional properties of these receptors in various brain regions. In response to the addition of a selective D_1-like agonist to an embryonic culture, neurons isolated from the MFC exhibit decreased spontaneous neurite outgrowth in a dose-dependent manner, while striatal neurons show increased process elongation. Conversely, selective D_2R activation promotes neuronal outgrowth in the cortex while inhibiting growth in the striatum[42-45]. Cultures isolated from the MFC of cocaine-exposed offspring exhibit greater spontaneous neurite outgrowth than neurons isolated from saline-exposed embryos. Cocaine-exposed cultures are also insensitive to the addition of exogenous D_1-like agonists to the culture indicating changes in responsiveness of the neurons to stimulation[43,46]. In coronal brain slices from cocaine-exposed progeny, permanent changes in the structure and trajectory of dopaminoceptive neurons in the ACC are visible. When quantified, a 40-50% increase in length of apical dendrites in layers III and V pyramidal neurons produce a characteristic "wavy" dendritic phenotype (**Figure 1a**)[40]. Increased length of dendrites is reflective of changes in local circuitry and loss of D_1R signaling which would normally serve to mediate inhibition of neuronal process development[43,46]. Similar structural abnormalities due to loss of D_1R signaling are exhibited in D_1R knock-out mice[47].

Investigating the underlying mechanisms contributing to diminished D_1R coupling following

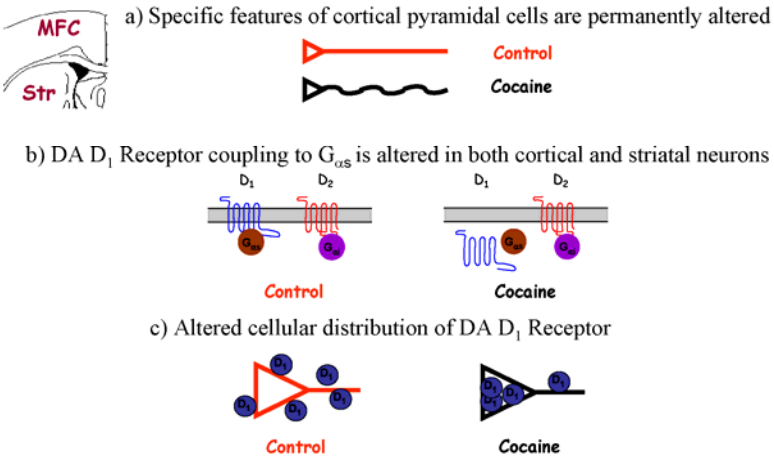

Figure 1 | **Prenatal cocaine exposure during a discrete period of embryogenesis results in long-lasting changes** in: **a** | cortical apical dendrite morphology, **b** | D_1R-$G_{\alpha s}$ coupling and **c** | D_1R subcellular distribution in cocaine exposed offspring.

prenatal cocaine exposure *in utero*, our laboratory established that there is a permanent reduction of DA-induced D_1R-$G_{\alpha s}$ coupling without a change in $G_{\alpha s}$ protein expression or total receptor density (**Figure 1b**). There is, however, a redistribution of the subcellular localization of the receptors such that fewer receptors are expressed on the plasma membrane (PM) while a larger proportion of receptors are permanently maintained within intraneuronal compartments[43]. Reduced surface expression of D_1Rs therefore reduces the number of receptors available for coupling to $G_{\alpha s}$ after agonist exposure (**Figure 1c**). The reduced coupling is specific for the D_1R - $G_{\alpha s}$ complex because coupling is not reduced for other $G_{\alpha s}$ coupled receptors, D_2R-$G_{\alpha i/o}$ coupling or muscarinic cholinergic receptors coupling to $G_{\alpha o}$ (in DA-rich areas)[46,48-49]. Disruptions in D_1R-$G_{\alpha olf}$ coupling have yet to be investigated. Alterations in signaling may be an adaptive response to the disrupted balance of DA and excessive receptor stimulation during development. Altered dopaminergic transmission has also been observed in other models of developmental modulation of DA content or receptor activation including dopamine depletion after denervation by 6-OHDA lesion[50] or TH inactivation[51], constitutive receptor activation in DAT knock-out mice[52-53], or selective modulation of signaling through D_1Rs or D_2Rs[54-55].

DOPAMINERGIC SIGNALING: REGULATION BY RECEPTOR AVAILABILITY

The availability of GPCRs at the PM is dynamically regulated by the neuronal environment including levels of neurotransmitter, intraneural trafficking and degradation. Modulation of the receptor density available for ligand binding is a key mechanism in the regulation of neuronal excitability and signaling. GPCRs are synthesized and folded in the endoplasmic reticulum (ER) before being transported to the Golgi apparatus where the proteins mature with addition of post-translational modifications. GPCRs are then targeted to the appropriate cellular membrane where they are able to interact with neurotransmitters[56-57]. The molecular mechanisms behind trafficking to the PM, receptor localization and surface expression are not fully understood, however, it is clear that D_1Rs exist as components of signaling complexes that can include channel proteins, other GPCRs, as well as scaffolding, cytoskeleton, and chaperone proteins[58-59]. DA receptor interacting proteins may be important regulators of D_1R transport from the ER and surface expression on the PM[60-61]. Specific post-translational modifications of the receptor are also implicated in regulating surface expression. Common modifications include glycosylation, palmitoylation, phosphorylation and ubiquitination.

N-linked glycosylation initiated in the ER and completed in the Golgi is the most common modification of GPCRs. Glycosylation involves the addition of oligosaccharides to specific asparagines residues with the consensus sequence NXS/T. The D_1R contains two consensus sites, one in the N-terminus region and the other in the second extracellular loop[16,62]. Receptor glycosylation might be important for D_1R PM localization and/or surface expression as it has been shown for other GPCRs[57], however, the data are conflicting regarding the role of D_1R glycosylation and PM expression[62-63]. D_1R glycosylation, however, is not necessary for ligand binding or coupling to G-proteins[63]. The D_1R has also been shown to undergo post-translational addition of fatty acid palmitate moieties at cysteine residues (Cys347 and Cys351) in the carboxyl tail of the receptor[64-65]. Palmitoylation of the receptor is likely involved in anchoring it to the membrane[16] as the majority of palmitoylated proteins are found at the PM[66]. As was shown with glycosylation of the receptor, it has also been shown that palmitoylation of D_1Rs is not involved in ligand binding or G-protein coupling[64]. Palmitoylation has also been shown not be involved with agonist-induced stimulation of AC or desensitization of D_1Rs[64].

Acute stimulation of receptors by DA reduces the number of receptors on the PM through a series of regulated processes, densensitization and internalization, for a period of time until removal of the ligand. Chronic stimulation, on the other hand, also reduces the number of receptors at the cell-surface but likely through different mechanisms of down-regulation. DA-mediated receptor activation promotes phosphorylation of the receptor at serine and threonine residues in the C-terminal region and third intracellular loop by receptor specific G-protein

coupled receptor kinases (GRKs) and cAMP-dependent kinases such as protein kinase A (PKA) activated by second messengers[67-70]. Phosphorylation of the receptor recruits binding of arrestin to the third cytoplasmic loop thus promoting uncoupling of the G-protein from the receptor. Arrestin targets the receptor to clatharin coated pits and recruits transport machinery for formation of the early endosome. Receptors are then internalized into intraneuronal compartments and de-phosphorylated by protein phosphatases before recycling back to the cell-surface in a resensitized state in which the receptors are competent to signal again. Alternatively, receptors can be trafficked to lysosomes or proteosomes for degradation[68-70]. Ubiquitination is an important modification made to receptors targeted for this pathway involving the covalent attachment of the small molecule, ubiquitin, to lysine residues of targeted proteins[71].

Alterations in post-translational modifications to DA D_1Rs are likely contributors to the underlying mechanisms behind the uncoupling of the receptor from $G_{\alpha s}$ and redistribution of the receptors after prenatal cocaine exposure. Changes in receptor modifications could potentially reduce delivery of receptors to the PM, thus keeping receptors sequestered in the ER or Golgi apparatus. Alternatively, aberrant modifications could increase the rate of receptor desensitization and internalization without proportional changes in resensitization. There is some evidence suggesting hyper-phosphorylation of receptors after prenatal cocaine exposure due to chronic receptor stimulation *in utero*. Receptor stimulation has been shown to increase phosphorylation of dopamine and cAMP-regulated phosphoprotein of 32kDa (DARPP-32) through activation of PKA. PKA phosphorylates DARRP-32 on Thr[34] thus converting it into an inhibitor of protein phosphatase 1 (PP1). PPI is responsible for de-phosphorylating many cellular substrates, thus decreased PP1 activity after cocaine exposure *in utero* could be responsible for maintaining receptors internally[72].

CONCLUSIONS

As has been reported, dopamine transmission modulates important events during development including neuronal process extension and establishment of normal circuitry. Insults to the dopaminergic system during development, such as chronic receptor stimulation *in utero*, result in permanent changes in dopaminergic signaling which may play a large role in the manifestation of neuropsychiatric disease states later in life. These alterations in signaling can result from the redistribution of receptors from the PM to internal compartments where they are no longer able to couple to G-proteins and mediate a response in the presence of ligand. Dissection of the molecular mechanisms behind alterations in receptor availability and subsequent changes in signaling cascades is relevant to understanding the pathophysiology behind diseases involving dysfunction of dopaminergic transmission whether it is hyper- or hypo-activity of the system. In fact, changes in dopamine receptor density have been observed in many diseased states. In schizophrenia, the density of D_2Rs has been shown to be elevated while the density of D_1Rs remains unchanged. In Parkinson's disease, increased D_1 and D_2 receptor densities has been shown to accompany loss of dopaminergic input into the midbrain. Similarly, loss of D_1 and D_2 receptor densities has also been observed in Huntington's disease patients. The studies proposed in the aims to follow are therefore not only important for understanding the normal and pathological states of the receptors but also in designing therapeutics for treating these disorders[73].

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FURTHER INFORMATION

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Structural Basis of Adhesion during Gastrulation and Brain Morphogenesis

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Morphogenesis, shape creation, is one of the central questions in developmental biology. It transforms a cluster of nearly identical cells in a blastula into a complex entity with structured tissues and organs. This transformation starts with gastrulation and reaches its highest complexity during brain morphogenesis. Molecular mechanisms driving morphogenesis have received great attention and cell adhesion molecules (CAMs) have been brought to the center of the stage. Here we review the structural basis of the functions of CAMs, highlighting their roles in gastrulation and brain morphogenesis. We also speculate the involvement of additional molecules such as adhesion G protein-coupled receptors (adhesion GPCRs) as novel CAMs in morphogenesis.

Blastula
An animal embryo, spherical in shape, composed of small cells derived from divisions of the fertilized ovum.

Gastrula
An animal embryo at the stage following the blastula. It is composed of three germ layers, the outer ectoderm, the middle mesoderm and the inner endoderm.

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Gastrulation is a masterpiece symphony performed by specified organ progenitors undergoing coherent morphogenetic movements. The movements of epiboly, internalization, convergence and extension transform the radially symmetric blastula into the gastrula with clear dorsal-ventral (D-V) and anterior-posterior (A-P) axes. Epiboly spreads the tissue vegetal-wards; internalization separates mesodermal and endodermal precursor cells from surface ectodermal layer; convergence drives tissue narrowing towards dorsal, and extension elongates the embryo anteroposteriorly[1]. After gastrulation, morphogenesis takes place within germ layers, tissues and organs and its complexity is championed by brain morphogenesis. Neural tissue starts out as a sheet of epithelium, which soon folds into neural tube. Within this structure, newly born neurons undergo migration to form cortical layers and cluster into functional groups. Most incredibly, synapses need to form precisely between two neurons among ten billions of neurons in the brain. Then, what's the mechanism underlying the powerful morphogenesis?

One major aspect of the answer goes to the cell surface. In 1955, Townes and Holtfreter prepared single-cell suspensions from each of the three germ layers of amphibian embryos soon after the neural tube had formed. By using embryos from species having cells of different sizes and colors, they were able to follow the behavior of cells from each layer, after cell suspensions were combined. Surprisingly, they found cells become spatially segregated after reaggregation and their final positions reflect their embryonic positions, with the ectoderm peripheral, the endoderm internal and the mesoderm in between[2]. This phenomenon can be nicely explained by "differential adhesion hypothesis" (DAH) proposed by Malcolm Steinberg. DAH reasons that the differences of the adhesive strength between cell types are what needed for sorting to occur, and the differential adhesive strength is endorsed by the differential expression of CAMs on the cell surface[3] (**Figure 1**).

There are five principal classes of CAMs: cadherin, immunoglobulin-like cell adhesion molecule (IgCAM), selectin, mucin, and integrin. Other molecules are also identified to possess adhesive properties, while mediating signal transduction. Ephrin and Eph are good examples of such molecules. Recently, a newly classified GPCR family, adhesion GPCR, has emerged as molecules with potential dual roles in cellular adhesion and signaling. Their functions in morphogenetic events are highly speculated.

STRUCTURAL BASIS OF ADHESION

Cadherins. Cadherins are characterized by the presence of cadherin repeats in their extracellular domain. Each cadherin has several tandem cadherin repeats and each of the 110-amino-acid repeats forms Greek-key β-sheet. The specific binding of three calcium ions between successive repeats rigidifies the extracellular domain to adopt an elongated crescent shape[4]. Cadherins are grouped into 5 subtypes, namely, classic cadherins, desmosomal cadherins, atypical cadherins, proto-cadherins and cadherin domain-containing proteins. The cytoplasmic domain of classic cadherins interacts with catenin complex, which anchors cadherins onto actin cytoskeleton.

In general, cadherins mediate intercellular adhesion via homophilic binding. Currently, domain-

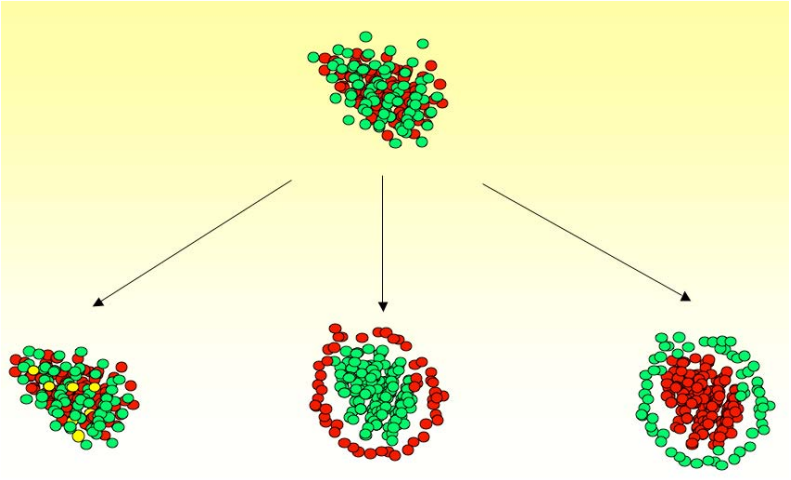

Figure 1 | **Demonstration of the DAH.** Green cells and red cells share the same cellular properties except the expression of CAMs. These two cell populations will stay in a mixture if the CAMs they express can bind to each other and endow cells with equal adhesive strength. Otherwise, the two cell populations will segregate. The more adhesive population will stay inside the less adhesive one.

swapping model is best supported by structural, biochemical studies on classic cadherins. In this model, two appose cadherins dimerize via their EC1 repeats (the distal most cadherin repeat). At the interface, the conserved Trp2 side chain from each molecule insert into the hydrophobic core of the other[5]. In support of this model, two CE1 monomer conformations were found in crystals: one with Trp2 side chain disordered[6] and the other, inactive, with the side chain inserted into its own hydrophobic pocket[7]. Furthermore, cis-dimer formation and clustering of E-cadherin have been shown to enhance its adhesive activity[8]. Not much is known about the binding of other cadherins, but differences from classic cadherins have been noted[9].

Cadherin-mediated cell sorting during morphogenesis has been an important question in the field. Early cadherin in-vitro transfection experiments suggested that the homophilic binding specificity determines sorting. However, later experiments argued that the quantity of surface-expressed cadherins determines the overall adhesive strength and is also important for cell sorting[10]. Furthermore, the observation of different conformational states of cadherin raised the possibility that cell signaling can regulate their adhesive activity.

IgCAMs. IgCAMs are CAMs with N-terminal immunoglobulin-like (Ig-like) domains. Like cadherin repeat, this Ig-like domain folds into a Greek key β-sheet. Depending on the number of β-strands, Ig-like domain can be subdivided into V-type (with 9 β-strands) and C-type (with 7 strands). The number of Ig-like domain contained in IgCAMs varies from 1 to 48. Likewise, members of this protein family have diverse mechanism of functions. Some have homophilic binding specificity, while others interact with other IgCAMs or other CAMs, such as integrin[11].

Via crystal structure studies, one common binding mechanism was found in several IgCAMs with homophilic binding specificity. IgCAM Hemolin has 4 Ig-like domains. In the crystal, these Ig-like domains bend into a horseshoe shape, with Ig-like domain 1 interacting with domain 4, and domain 2 with domain 3. Therefore it was speculated that, when two Hemolin proteins come close from opposing membranes, the Ig-like domain 1 and 2 of one Hemolin could bind to Ig-like domain 4 and 3 of the other Hemolin and vise versa[12].

One of the special traits of IgCAMs, in regards to differential adhesion, is their impressive repertoire of splicing variants. One extreme example is *Down syndrome CAM (DSCAM). DSCAM* can potentially be spliced into 38016 isoforms in *Drosophila.* Recently, the crystal structures of the Ig-like Domains of two *DSCAM* isoforms were determined. Interestingly, the different peptides generated by alternative splicing in domain 2 and 3 were pivotal to determine the homophilic binding specificity. Swapping these peptides could completely switch the binding specificity between these two isoforms[13].

Integrins. Integrins are heterodimers of two single-transmembrane subunits (α and β). There are 18 α subunits and 8 β subunits encoded in vertebrate genomes, forming at least 24 different integrins. Integrin molecules can be dissected into 3 parts: the cytoplasmic region, the membrane-proximal tailpiece and the membrane-distal headpiece. Ligand-binding specificity of integrins is encoded in the I domain (of some α subunits) or the I-like domain (of β subunits) in the headpiece[14].

Integrins can exist in different ligand-binding affinity states, corresponding to different confirmations. In the low-affinity state, the tails and cytoplasmic regions of α and β subunit associate with each other to restrain the headpiece in a bent conformation. When integrins shift into the high-affinity state, the headpiece dissociate from tailpiece to adopt an extended confirmation[15]. This shift can be induced by the presence of extracellular ligand and inside-out signaling.

The diverse roles of integrins during morphogenesis, in part, come from their ability to mediate cell-cell and cell-matrix adhesion. Integrins are able to form heterophilic interactions with multiple CAMs and cell matrix proteins, such as IgCAM, E-cadherin, fibrinogen, collagen and laminin. *Mucins and selectins.* Interactions of selectins and mucins mediate tethering and rolling adhesion of leukocytes and platelets on vascular surfaces.

Selectins are transmembrane proteins with a membrane-distal lectin domain, which binds to sLe^x on the mucin side chain in a Ca^{2+} dependent manner[16]. On the other hand, mucins are large, heavily glycosylated proteins. Their serine- and threonine-rich mucin motif is subject to extensive O-glycosylation, which decorates the main peptide chain like a bottlebrush[17]. The cytoplasmic regions of both mucins and selectins are anchored to actin skeleton.

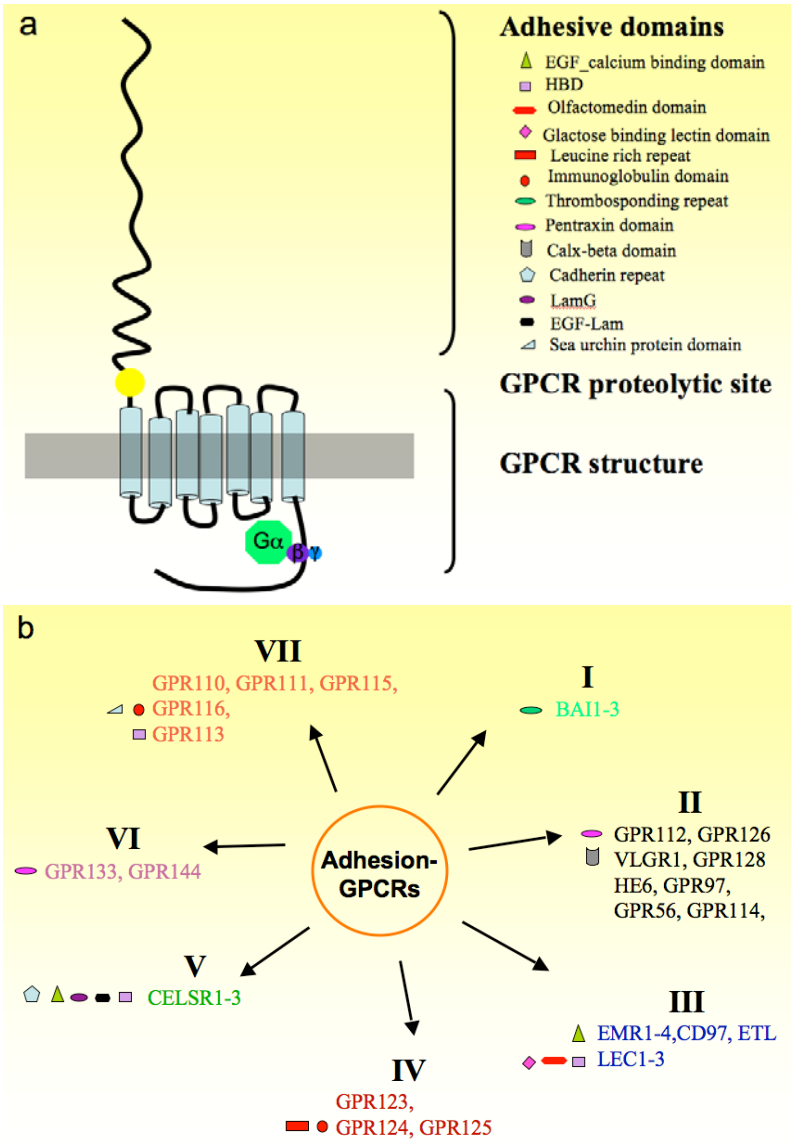

Figure 2 | **Schematic protein structure and subfamilies of adhesion GPCRs. a** | Adhesion GPCRs are natural chimeras of adhesion molecules and GPCRs. After the cleavage within the GPCR proteolytic site, the extracellular fragment and the GPCR fragment form heterodimers via non-covalent bonds. **b** | Human adhesion GPCRs are divided into seven subfamilies based on the phylogenetic study of their seven-transmembrane domain. Members of one subfamily tend to have same adhesive domains. BAI, the brain-specific angiogenesis-inhibitory receptor; VLGR, the very large G-protein-coupled receptor; HE6, Human Epididymis-specific protein 6; EMR, the EGF-like module containing receptor; ETL, the EGF-TM7-latrophilin-related receptor; LEC, lectomedin receptor; CELSR, the EGF LAG seven-pass G-type receptor.

Deletion mutants, lacking the binding sites for cytoskeleton proteins affect or eliminate rolling adhesions[18]. Interestingly, mucin-like motif is frequently seen in adhesion GPCRs. It adds potential adhesive value to adhesion GPCRs.

Ephrins and Ephs. Ephs are receptor tyrosine kinases with distinctive extracellular features. Their extracellular region, comprised of an N-terminal ephrin binding domain, an EGF-like domain and two fibronectin III motifs, is reminiscent to other CAMs. Their ligands, ephrins, are grouped into two classes: EphrinAs anchor on the plasma membrane through a glycosylphosphatidylinositol group, while EphrinBs have a transmembrane and cytoplasmic domain. Accordingly, EphrinA-binding Ephs are called EphAs, and EphrinB-binding Ephs are EphBs.

According to the crystal structure of EphB2 and EphrinB2 complex, each Eph bind to an ephrin through an expansive dimerization interface dominated by the insertion of an extended ephrin loop into a channel at the surface of the receptor. Then two Eph-Ephrin dimmers join to form a ring-like tetramer[19]. This high-affinity binding can be switched off via two mechanisms. It was discovered that interaction of EphA3 with EphrinA2 or A5 leads to cleavage of the ligand by ADAM-10 metalloproteinase, resulting in the dissociation of ligand from receptor[20, 21]. In addition, EphB-EphrinB interaction can be terminated by endocytosis of the complex into EphB- or EphrinB- expressing cells[22, 23].

The manifest effect of ephrins and Eph receptors during embryonic morphogenesis is to mediate cell segregation at the interface of their complementary expression domains or within regions of co-expression or overlapping gradients[24]. This effect provides striking example for DAH that cells with different adhesion properties would adjust their positions to maximize their bindings with cells of similar affinity.

Adhesion GPCRs. Before Adhesion GPCRs were given this name, some of them were known as LN-TM_7 or EGF- TM_7 receptors, implying that they are seven transmembrane proteins with EGF-like domains in the long extracellular N-termini. Since they are most related to secretin-receptor family (B1) in sequence, these receptors were classified as B2 family GPCRs[25]. However, the overall sequence similarity between these LN-TM_7 receptors and B1 receptors is fairly low and they differ in many aspects. In 2002, Fredriksson *et al*. proposed a new GPCR classification system, GRAFS, based on the phylogenetic analysis of the entire repertoire of the seven transmembrane regions of GPCRs. In GRAFS, LN-TM_7 receptors were for the first time grouped into a distinct family and named as adhesion GPCR[26].

GRAFS
Glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin: The five main GPCR families.

Morpholino oligonucleotides
Modified antisense oligonucleotides, which bind to the complementary RNA sequences to block RNA splicing or translation.

Later, the same group created Hidden Markov Models derived from GRAFS groups to survey the genomes from 13 species[27]. They found adhesion GPCRs exist in all animal species surveyed and there are 33 human adhesion GPCRs and about 22 zebrafish adhesion GPCRs, which is in the same range with our findings (**Figure 2**).

The long N-termini of Adhesion GPCRs are usually composed of several functional domains[28]. GPCR proteolytic site (GPS) is conserved in all Adhesion GPCRs, except *GPR123*. It is located adjacent to the first transmembrane region and contains 4 conserved cystines, one glycine and two tryptophans. The cleavage within GPS has been reported for CD97, ETL, EMR2, EMR4 and LEC1 and it was shown that the cleavage is essential for surface expression of LEC1[29]. After cleavage, the two parts form a heterodimer via non-covalent interaction. Other than GPS, adhesion GPCRs have functional domains with adhesive properties, such as cadherin repeat, EGF-like domain, Ig-like domain, leucine-rich domain and mucin-like motif (**Figure 2**). Very little is known about the interaction of adhesion GPCRs with their ligands. Nevertheless, CD97 was reported to bind the SCR repeat pf CD55 via its first two EGF-like domains[30], while bind chondroitin sulphate via its forth EGF-like domain[31]. Since more than half of adhesion GPCRs have multiple adhesive domains and all of them are highly glycosylated, they are likely to interact with more than one ligand.

So far, only GPR56 has been shown to functionally couple to $G\alpha_{12/13}$[32] and form a complex with $G_{q/11}$[33]. But G protein–coupling to other adhesion GPCRs remains a possibility. Other intracellular interacting proteins were discovered for some adhesion GPCRs. The combination of unique features supports the notion that adhesion GPCRs could act as adhesion molecules with signaling capability.

ADHESION AND GASTRULATION

From studies on zebrafish, we learned that gastrulation movements are driven by a variety of cell behaviors. Slow- and fast- directed migration and mediolateral intercalation drive convergence and extension; radial intercalation plays an important role in epiboly of deep cells, and cell movements are coupled with changes of cell shape[1]. The contributions of CAMs to these behaviors are indispensable.

E-cadherin plays widespread roles during zebrafish gastrulation. Mutations in *half baked (E-cadherin)* cause epiboly arrest, disrupted convergence & extension and failure of prechordal plate cells to elongate and migrate efficiently towards animal pole after internalization. Kane *et al*. reported that there is a radial gradient of E-cadherin expression from the deepest layer of the blastoderm (lowest expression) to the superficial layer of the blastoderm (highest expression) at shield stage. They reasoned upregulation of E-cadherin was required to maintain cells in the exterior layer after radial intercalation, since in *half baked* mutant, radially intercalated cells tend to neither change cell shape nor become restricted and often de-intercalate and move back to the interior layer[34]. By contrast, Montero *et al*. argued that embryos, injected with E-cadherin morpholino oligonucleotides to block E-cadherin expression, had reduced radial intercalation at 65% epiboly[35]. Although these two reports seemingly failed to reach a consistant conclusion, they in fact suggest that perfect strength of E-cadherin mediated adhesion is required for normal gastrulation and its slight changes might lead to different types of cell-behavioral defects.

The crosstalk between CAMs during gastrulation is another outstanding question in the field. It was first shown that protocadherin could regulate *Xenopus* gastrulation via homophilic interactions. However, Chen and Gumbiner later found more compelling evidence that paraxial protocadherin (PAPC) mediates cell sorting and influences gastrulation movements by down-regulating C-cadherin activity in *Xenopus* embryos. Among other lines of evidence, they found a dominant–negative form of C-cadherin can rescue the blastopore closure defect, caused by loss of endogenous PAPC[9]. Interestingly, crosstalk between CAMs from different families was also reported. Marsden and Desimone discovered that applying fibronectin blocking antibody or expressing a dominant- negative form of β1 integrin alters C-cadherin-mediated cell adhesion and inhibits medial-lateral cell intercalation and axial extension in gastrulating *Xenopus* embryos and explants[36]. The same group also reported that fibronectin and integrin interaction suppresses random protrusions in favor of polarized protrusions to facilitate mediolateral intercalation[37]. However, whether C-cadherin is involved in this process was not mentioned. Although the detailed mechanisms of crosstalks are still elusive, it is confirmed that C-cadherin expression level is not altered in either case. As we dig deeper, more adhesion molecules and more adhesive crosstalks ought to be discovered in the future.

ADHESION AND BRAIN MORPHOGENESIS

CAMs play diverse roles in nearly all aspects of brain morphogenesis, from neurulation to synaptogenesis. Their roles in brain morphogenesis are implied by their distinctive temporal and spatial expression pattern in the brain and justified by the phenotypes of knockout, knockdown, mutant animal models or human diseases. Among cadherins, the function of N-cadherin in the developing nervous system has been extensively studied. In zebrafish, it is

required to maintain the integrity of neuroepithelium[38] and it also plays a role in axon migration[39]. Differential combinatorial expression of type II cadherins could regulate motor neuron pool sorting[40]. And differential and combinatorial expression of protocadherins is also speculated to play a role in establishing specific neuronal connections, based on the existence of multiple splicing variants and their synaptic localization. An exciting progress has recently been made on *DSCAM*, of which the outrageous alterative splicing was mentioned above. Using mosaic analysis to mark single neurons, homophilic DSCAM-DSCAM interactions are demonstrated to be required for dendrite self-avoidance in *Drosophila* larval[41]. Another long-term favored subject in this field is the function of ephrins and Ephs during rhombomere formation. Several ephrins and their corresponding Ephs are found expressed in alternating presumptive rhombomeres. Lines of evidence demonstrate that ephrin- and Eph-mediated repulsion at rhombomere interface drives cell sorting and boundary formation[42]. However, it is not the only way Eph signaling regulates rhombomere formation. When Cooke *et al*. transplanted EphA4 morpholino-injected (MO) cells into wild-type (WT) embryos, they found that those cells could integrate with host cells in even-numbered rhombomeres (which don't express EphA4), while these transplanted cells were pushed robustly to the edges of r3 and r5 (both of which express EphA4). Reversely, when WT cells were transplanted into EphA4MO embryos, they formed pure clusters within r3 and r5. In both cases, transplanted cells maintained r3 or r5 identities within r3 and r5[43]. This experiment suggests that Eph- mediated cell adhesion within rhombomeres also contributes to cell sorting during rhombomere formation.

Rhombomere
A transiently divided segment of the developing neural tube in the area of future hindbrain.

A new aspect of the field is opened by the emergence of adhesion GPCRs. Although the majority of adhesion GPCRs are still poorly studied orphans, the critical functions of *Celsr* proteins and *GPR56* during brain development have been unveiled. *Celsr* genes are mammalian homologues of *Drosophila flamingo*. The homozygous *Celsr1* mutant embryos fail to initiate neural tube closure and have severe defect in the planar cell polarity of hair cells in the organ of corti[44]. Celsr2 and Celsr3 regulate neurite growth in an opposing manner. Celsr2 enhanced neurite growth, whereas Celsr3 suppressed it[45]. In addition, Celsr3 mediates axonal tract formation in mammals[46]. It was also uncovered that Celsrs regulate facial motor neuron migration in zebrafish[47]. The mutations in *GPR56* were first identified from patients with bilateral frontoparietal polymicrogyria[48]. Consistently, loss of *Gpr56* function in mouse results in a cobblestone-like cortical malformation[49]. Li *et al*. provided compelling evidence that GPR56 interacts with a yet unidentified ligand in the marginal zone or overlying extracellular matrix to regulate the integrity of pial basement membrane and therefore influence cortical lamination. Comparable expression profile of each adhesion GPCR has been studied via RT-PCR in mouse and rat[50]. More than half of them show predominant expression in the nervous system. In addition, the in-situ hybridization data of several adhesion GPCRs in early zebrafish embryos have been reported[51]. These initial discoveries suggest that the research in the field is still at its infancy stage and more exciting discoveries are still to come.

CONCLUSIONS

By far, we have gained deep structural insights into homophilic or heterophilic interactions between CAMs. It substantially facilitates our understanding of their functions in various biological processes, including gastrulation and brain morphogenesis. The knowledge gained from these studies can guide our studies on novel molecules with similar functional domains and the techniques created for these studies can be further applied to new studies. My research project will focus on the roles of novel adhesion GPCRs in zebrafish gastrulation and brain morphogenesis. Adhesion GPCRs possess unique structural assets. They have diverse functional domains with adhesive properties and the characteristic seven transmembrane region of GPCR. Their enriched expression in the nervous system and early expression in zebrafish embryos indicate their function during gastrulation and brain morphogenesis. Furthermore, *Celsr* and *GPR56* have been shown to play important roles during gastrulation and brain morphogenesis. It again invites investigations on other members of this GPCR family.

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FURTHER INFORMATION

Heidi Hamm's Lab: http://pharmacology.mc.vanderbilt.edu/Faculty/Hamm_Lab/research.php
Lila Solnica-Krezel's Lab: http://sitemason.vanderbilt.edu/site/jSIHBu/HomePage

The Zebrafish Habenulae: Abundant Asymmetry in the Dorsal Diencephalon

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The animal kingdom abounds with examples of asymmetric body plans. In mammals, a gross examination of the visceral organs reveals many of these asymmetries, including the biased placement of the heart, liver, and pancreas along the Left/Right (L/R) axis. A discussion of asymmetry is easily extended to the beautifully complex organization of the brain. For instance, the human cerebral cortex has evolved specialized regions that are specific to one hemisphere; Broca's area, the locus for speech, is found specifically in the left hemisphere in the vast majority of individuals. In fact, brain asymmetry is quite common throughout the vertebrate lineage, suggesting that lateralized organization is of evolutionary merit and thus contributes adaptive advantages. Although it is difficult to correlate molecular deviations in symmetry with functional consequences in a behaving animal, careful characterization of asymmetrical brain development may eventually unveil the essential components of the nascent lateralized brain.

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The habenular nuclei, a model system for studying laterality in an emerging molecular brain, serve as relay stations of the dorsal diencephalic conduction pathway. A broad discussion of the habenulae across vertebrates is a challenging one, as functional studies are sparse, and the conservation of connectivity does not necessarily hold between divergent species. In mammals, the habenular nuclei represent a vital transit center of limbic processing, and accordingly, they have been implicated in a variety of cognitive and behavioral studies, but these limbic connections are not present in lower vertebrates. From a laterality perspective, the most intriguing aspect of the habenulae is witnessed through their asymmetric development in some fish, reptiles, and amphibians. Many studies have established the dorsal diencephalon, or epithalamus, as a premier locus of study for brain asymmetry[1]. In particular, the zebrafish has emerged as a premiere system for genetic and molecular developmental studies of the epithalamus, and these methods have revealed distinct habenular asymmetries.

VERTEBRATE DORSAL DIENCEPHALON

The dorsal diencephalon (epithalamus) of vertebrates contains a paired set of habenulae along with a photoneuroendocrine pineal organ, with the addition an accessory organ, termed the parapineal, parietal eye, or frontal organ, in fish, reptiles, and amphibians respectively[1]. There are many variations as to the specific organization of the epithalamus, but in general the pineal organ is situated at the midline of the brain, flanked by the habenulae. When present, the parapineal is often biased to one side of the brain, and this accessory nucleus provides a stark example of brain asymmetry (**Figure 1**). The parapineal organ has been shown to innervate the left dorsal habenula in trout[2], and lamprey[3], and more recently in zebrafish as well[4-6].

Research on the mammalian habenulae prompted Sutherland to describe the dorsal-diencephalic conduction pathway, in which the habenular nuclei serve as a relay center from limbic forebrain to midbrain[7]. On either side of rat brain exist distinct lateral and medial divisions of each habenular nucleus. Indeed, in all mammals, the medial (MHb) and lateral nuclei (LHb) have marked differences in their respective afferents and efferents[7-12]. It has been proposed that the medial habenula of lizards and mammals is homologous to the habenula of lampreys and teleosts[2,3,13], due largely to the fact that habenular efferents from said nuclei directly target the interpeduncular nucleus (IPN)[14-16], as do the majority of fibers from the mammalian MHb[9-11]. Thus this connection to the IPN via fasciculus retroflexus (FR) is highly conserved[8]. In fact, utilizing tritiated amino acid injections in various regions of rat habenulae, followed by autoradiography, Herkenham and Nauta conclude that no LHb projections appear to involve the IPN, in the rat[11]. Interestingly, it should be noted that afferent connections to the zebrafish habenulae are fairly homologous to those reaching the rat lateral habenulae, with many fibers originating in the eminentia thalami (EmT) or entopeduncular nucleus, respectively[12,17]. For simplicity, discussion here will be limited to the MHb in rat.

The main inputs to the medial habenula originate from the septum, and their afferent path is through the stria medullaris (SM)[7]. In fact, horse radish peroxidase (HRP) injections in the medial habenular nucleus show that only neurons in the supracommissural septal area contribute to the SM[12]. There are also ascending inputs to the medial habenula, as both serotonergic fibers from raphe nuclei[12, 18] and noradrenergic fibers from the dorsolateral tegmental nucleus and ventral central gray target the region[19]. Noradrenergic afferents travel successively through the dorsal tegmental bundle, medial forebrain bundle, and SM[19]. Thus both septal and midbrain nuclei provide afferent innervation of the MHb.

Efferents from the medial habenula project to the interpeduncular nucleus via the fasciculus retroflexus (FR)[9-11]. The rat medial habenula possesses segregated populations of neurons of both acetylcholine and substance P neurochemical nature, and these neurons retain exclusive targets in the IPN: cholinergic terminals have been found in the central core of the IPN, and substance P projections appear to innervate the periphery[20,21]. In fact, chronic exposure to nicotine causes axonal degeneration of the FR in rat, presumably through nicotinic ACh receptors[22]. Other efferents through the core of the IPN are glutamateric[23]. All habenular efferents through the fasciculus retroflexus are distinctly segregated: the core (central) processes stem from the medial habenula and the mantle (peripheral) from the lateral habenula[11]. Additionally, there may also be minor projections from the medial habenula to the ventral tegmental area as suggested from a lesion study[24].

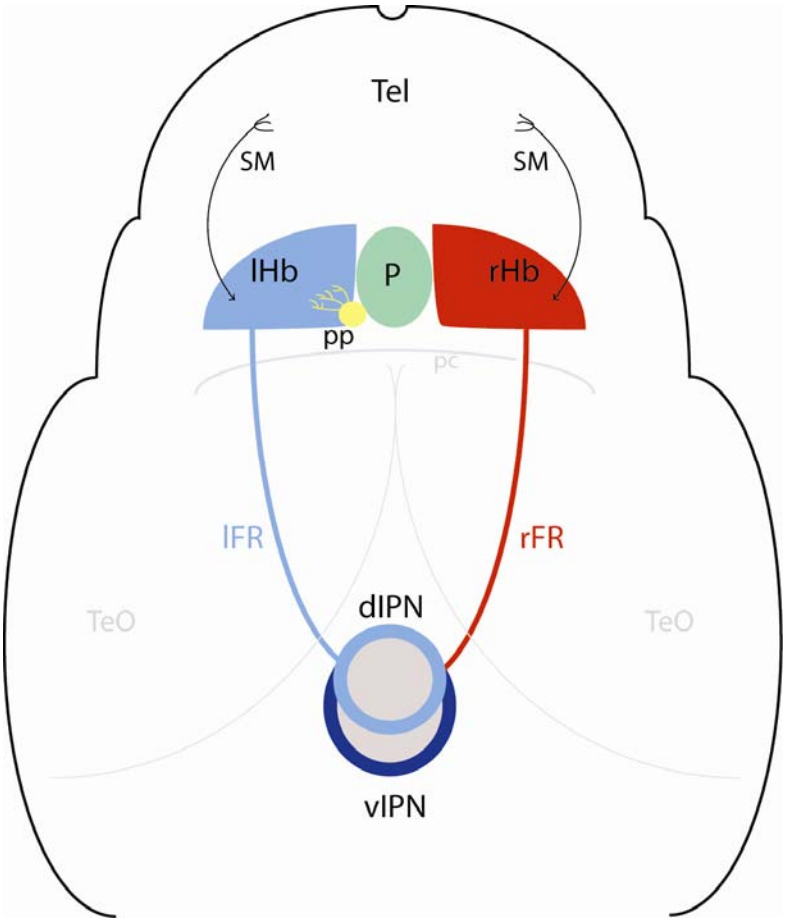

Figure 1 | **Dorsal view of a larval zebrafish at 4 days post-fertilization**. The larval epithalamus exhibits distict asymmetries. The pineal organ (P) exists as an emanation from the roofplate at the midline. The parapineal (pp) is situated both left and rostral to the pineal, and is situated just caudal to the left habenula (lHb). The habenulae themselves exhibit differences in overall size and volume of dense neuropil (yellow efferents from pp). Also shown is input from forebrain via stria medullaris (SM), and laterotopic output through the fasciculi retroflexus (FR), terminating in the interpeduncular nucleus (IPN). The ventral IPN receives input from left and right habenulae. pc, posterior commissure; TeO, optic tectum. *Adapted from* [6].

Habenular function has been implicated in a variety of behaviors, as can be deduced from their diverse connectivity, yet many of these correlations are specific to the lateral habenulae. In regard to the medial habenulae, functional studies implicate feeding and mating[7], as well as hormone secretion[25]. Additionally, several mammalian studies have implicated the lateral habenula in psychosis[26], addiction[23], avoidance learning[27,28], and as a source of negative reward signals on dopaminergic neurons[29]. These studies implicating the lateral habenula via fasciculus retroflexus likely reflect inhibitory influence on dopaminergic neurons[23], as lesions of the SM, LHb, or FR increase dopamine turnover in prefrontal cortex, nucleus accumbens and striatum[30, 31]. Even so, it should be noted that the lateral habenula does not project to the IPN, and thus these higher order cognitive functions may not be conserved in zebrafish.

As noted above, there are significant differences in the basic organization of the dorsal diencephalon across the vertebrate clade. While subtle differences between left and right habenulae have been noted in the albino rat[32] and albino mouse[33], as well as a sex-specific difference in medial habenula in chick[34], more ancient vertebrate lineages present more explicit examples of asymmetry. For instance, the hagfish, lamprey, eel, newt[35], frog[36], and lizard[37] all show dramatic habenular asymmetry. Even so, the basic organization and connectivity of this region remains comparable across the vertebrate lineage. Thus, there is likely substantial conservation of the genetic programs responsible for epithalamic development.

ASYMMETRIC HABENULAE OF THE ZEBRAFISH (*DANIO RERIO*)

The zebrafish habenular nuclei display striking asymmetries in connectivity, nuclear organization, and gene expression, all of which result from an asymmetric developmental program. The first, and most subtle, is a slight leftward bias of the pineal organ stalk from the roofplate of the dorsal diencephalon[38]. More obvious is the placement of the accessory parapineal, which arises from a common pool of progenitor cells within the pineal complex as shown by lineage labeling at 22-24 hours post fertilization (hpf)[4] and more elegantly through time-lapse imaging[39]. By 28 -32 hpf, it is a distinguishable organ, with a left-bias in around 95% of embryos[5,14].

Finally, the habenulae show several L/R differences. First, anti-acetylated tubulin labeling demonstrates more dense volume of neuropil in the left habenula[4] (**Figure 1**). Secondly, *in situ* hybridization of *cpd2*, a gene expressed bilaterally in the habenulae, shows an 18% greater area in the left habenula of larvae at 4 days post-fertilization (dpf). Finally, an additional set of marker genes, which contain a *potassium channel tetramerization domain* (*kctd*), also show distinct asymmetries. *leftover* (*kctd12.1*), is more widely expressed in the left habenula[14], whereas the remaining two, *right on* (*kctd12.2*) and *dexter* (*kctd8*), are increased on the right[15].

In recent years, the connectivity of the zebrafish dorsal diencephalon has been well characterized. Lipophilic dye tracing and has provided information on afferent connections. The majority of habenular innervation derives from migrated neurons from the EmT[17]. Innervation of the habenulae by migrated EmT neurons or the adult entopeduncular nucleus, via the stria medullaris, is conserved across species, in trout[13], goldfish[40], and rat[41]. In addition, neurons from the pallium (dorsal telencephalon) and posterior tuberculum (diencephalon) provide input[17,42]. Interestingly, in zebrafish, pallial projections are asymmetric as they terminate in the right medial habenula despite their side of origin[17]. In addition, antibody labeling against SV2, a presynaptic glycoprotein, demonstrates that neuropil density is higher in the left lateral habenula, and unveils a unique extension to the right medial subnucleus[17]. These results demonstrate that afferent innervation is also asymmetric and may contribute to the development of these lateralized nuclei.

The asymmetric habenulae of zebrafish appear to be coupled to laterotopic innervation of the IPN, the primary efferent target. Anterograde tracing studies using the lipophilic dyes DiO and DiI for left and right habenula respectively, demonstrate that left and right FR have different projection patterns and specific targets: efferents from the right habenula innervate the ventral region of the IPN, whereas the left habenula projects primarily to the dorsal region[14, 43]. Additionally, immunolabeling for Leftover and Right on proteins serve as specific tracers of left and right habenular efferents, respectively. Leftover positive axons (Lov+) target the dorsal and ventral regions of the IPN, whereas Ron+ axons are restricted to the ventral IPN[15]. In a developmental perspective, these Lov+ growth cones reach the IPN by 2 dpf, and habenulo-interpeduncular connections are well formed by 4 dpf[15].

MOLECULAR AND GENETIC CONTRIBUTIONS TO ASYMMETRIC HABENULAR DEVELOPMENT

Early patterning events in the embryonic zebrafish have a profound effect on habenular asymmetry. Mutational analysis of the Nodal genes indicate that they impact the stereotypic laterality of both visceral organs and the central nervous system[44]. In addition, misexpression of Nodals can lead to altered L/R polarity of organs[45]. The Nodal cascade is not necessary for the development of asymmetry, but is essential to determine the direction of laterality. For example, *one-eyed pinhead* mutants, which have a deficiency in the Nodal receptor complex, must be rescued past an early gastrulation requirement for Nodal by injection of *oep* RNA (these embryos are referred to as rescued *oep*, or R*oep*). These embryos are viable, yet show randomized epithalamic organization: the parapineal and ipsilateral, enlarged habenula are on the left and right sides at equal frequency[4, 38].

This randomization, as assessed by neuropil density and *leftover* expression is also seen through disruption of the earliest acting Nodal ligand, *southpaw*[46]. Targeted morpholino knockdown of *southpaw* transcript results in L/R randomization of parapineal migration, and leads to disruption of IPN targeting: when the parapineal is situated at the right efferents from left habenula project solely to ventral domain, while right habenula afferents project along entire dorso-ventral axis[15]. Early Nodal signaling is thus crucial to establish polarity of both visceral organs and the CNS.

There is substantial evidence that the asymmetric development of the habenulae in zebrafish is dependent on the parapineal. As stated previously, the parapineal is morphologically apparent at 26 hpf, while the first expression of *leftover* cannot be detected until between 38[14]. This accessory nucleus innervates a circumscribed, central region of the ipsilateral habenular nucleus[4-6]. This central habenular region is also coincident with a described zone of enlarged neuropil[4](Gamse, unpublished). In summary, the left habenula is larger, possesses denser neuropil, and is innervated by the left-sided parapineal, a situation that is reversed in embryos with right-sided parapineal placement (*spaw* morpholino injected embryos)[15].

Cell ablation studies provide a more direct examination of the relationship between the parapineal and habenular asymmetry. When the cells of the parapineal are ablated soon after beginning migration, the left habenula no longer develops asymmetrically. More specifically, the left and right habenulae now resemble each other in volume of neuropil and expression of *leftover*[5;14]. Specifically in regard to the left habenula, ablation also results in expanded expression of *ron* and *dex*[15]. In fact, in parapineal ablated larvae, all three *kctd* genes appear bilaterally symmetric, with subdomains typical of a wild type right habenula[15]. These results suggest that in the left habenula, the parapineal may be responsible

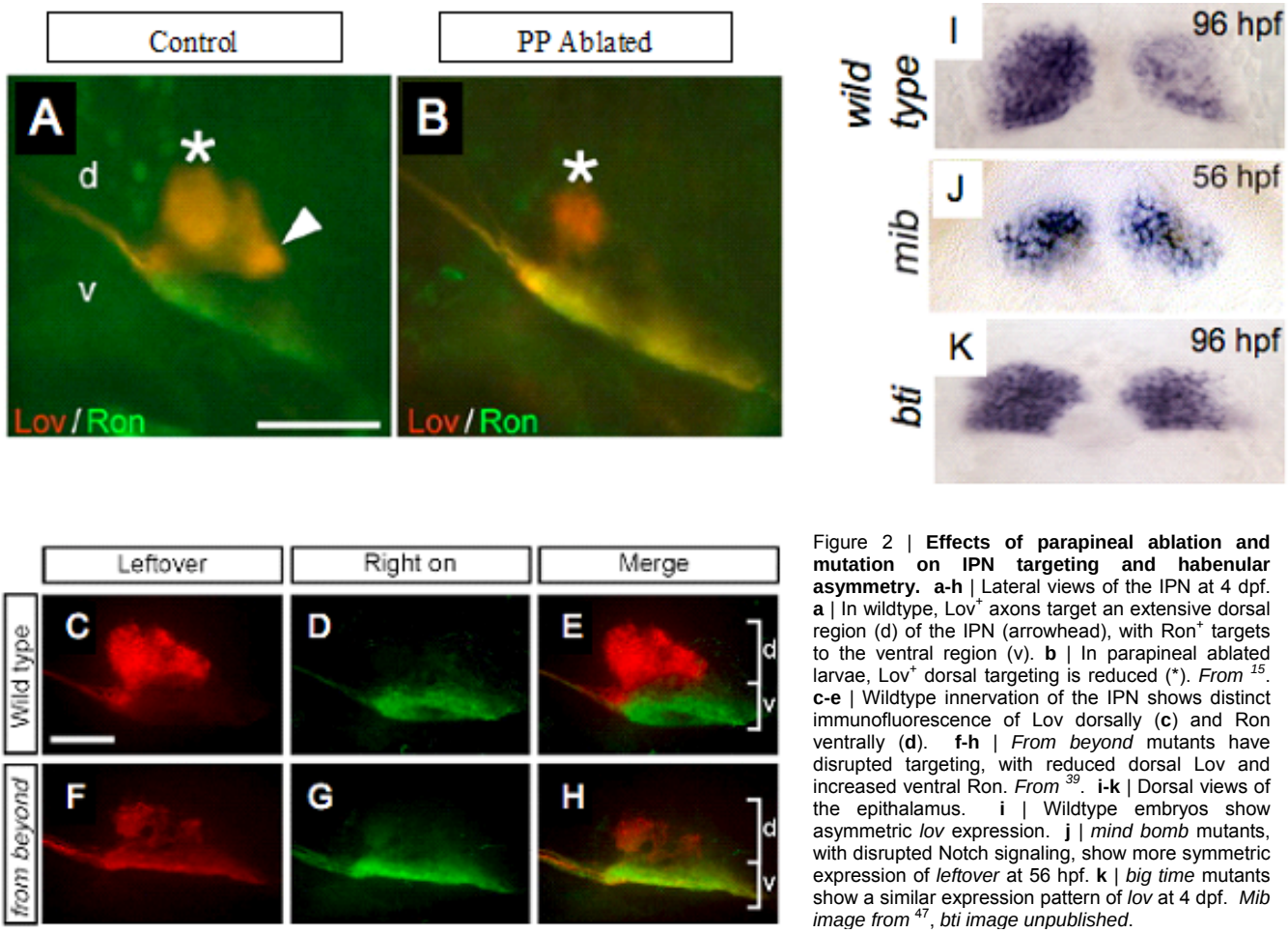

Figure 2 | **Effects of parapineal ablation and mutation on IPN targeting and habenular asymmetry. a-h** | Lateral views of the IPN at 4 dpf. **a** | In wildtype, Lov$^+$ axons target an extensive dorsal region (d) of the IPN (arrowhead), with Ron$^+$ targets to the ventral region (v). **b** | In parapineal ablated larvae, Lov$^+$ dorsal targeting is reduced (*). *From* [15]. **c-e** | Wildtype innervation of the IPN shows distinct immunofluorescence of Lov dorsally (**c**) and Ron ventrally (**d**). **f-h** | *From beyond* mutants have disrupted targeting, with reduced dorsal Lov and increased ventral Ron. *From* [39]. **i-k** | Dorsal views of the epithalamus. **i** | Wildtype embryos show asymmetric *lov* expression. **j** | *mind bomb* mutants, with disrupted Notch signaling, show more symmetric expression of *leftover* at 56 hpf. **k** | *big time* mutants show a similar expression pattern of *lov* at 4 dpf. *Mib image from* [47], *bti image unpublished*.

for specifying neurons that show greater expression of *leftover*, and may have a role in repressing right-sided gene expression[15]. In addition, these embryos have fewer Lov+ axons, and their projection within left fasciculus retroflexus (FR) now resembles that seen in the right FR. The IPN target is also affected, in that Leftover immunofluorescence is visible only in one small anterior domain in the dorsal IPN, with a concurrent increase in ventral IPN targeting[15] (**Figure 2a-b**).

Finally, a mutant analysis provides further evidence of the impact of the parapineal on asymmetric habenular development, and resultant connectivity to the midbrain. The *from beyond* (*fby*) mutation, mapped to the *tbx2b* gene, results in a nearly complete reduction in parapineal cells in embryos homozygous for the lesion. These embryos also demonstrate a habenular phenotype of symmetric expression genes *leftover*, *right on*, and *dexter*[39], as well as a reduction in Lov+ targeting to the dorsal region of the IPN, and a concordant increase in Ron+ targeting of the ventral IPN[39] (**Figure 2c-h**). This mutation, effectively blocking the formation of the parapineal organ, highlights the dependency of the habenulae on the parapineal, and shows a disruption in the dorsal diencephalic pathway through altered targeting of the midbrain nucleus, which is reminiscent of parapineal ablated larvae.

The expression of several habenula-specific genes in the zebrafish dorsal diencephalon suggests the existence of distinct medial and lateral subnuclei. It should be noted that this subnuclear division is not equivalent to the medio-lateral division in mammals, which have more distinct divisions and connectivity. In zebrafish, *leftover*, *right on*, and *dexter* visibly label different regions of the habenulae, with distinctions on dorso-ventral, medio-lateral, and antero-posterior axes[15]. In addition, the *brn3a* promoter drives expression of green fluorescent protein (GFP) specifically in the medial habenula[16]. Expression of these genes suggests that the right medial habenula is larger as compared to left. Conversely, the left lateral habenula is larger than the right[47]. In fact, these cell populations possess distinct neurogenetic programs: birth date analysis shows differential timing of neurogenesis between L/R and medio-lateral cell groups[47].

Thus far, we have witnessed the impact of early

Nodal signaling and parapineal placement on the developing asymmetric habenulae of zebrafish. There is an implied relationship between the parapineal and left habenula, yet that message has not been elucidated. Ultimately, asymmetric habenular nuclei must arise from a carefully controlled program of neurogenesis. The Okamoto laboratory took steps to characterize this neurogenesis through detailed birth date analysis, utilizing incorporation of 5-bromo-2-deoxyuridine (BrdU) in embryos expressing GFP under the *brn3a* promoter[47]. These embryos express GFP specifically in the medial habenular subnuclei, allowing medio-lateral distinction. Embryos were pulse labeled with BrdU at various developmental stages, and then allowed to develop to 5 dpf. They found that GFP- lateral habenula neural precursors were born first, beginning at 24 hpf, and peaking at 32 hpf. Neural precursors for the GFP+ medial habenula were born later, with a few visible at 32 hpf and a peak at 48 hpf. There was a significant difference in BrdU+ cells in left versus right habenula as early as 32 hpf, and significantly more medial habenular cells were born in the right habenula at 48 hpf. In short, there were more early-born lateral habenula cells on the left and more late-born medial habenula cells in the right[47], but the signaling mechanism responsible for the timing of habenular neurogenesis has yet to be determined.

One obvious candidate for such a mechanism is Notch signaling, which has been implicated in the maintenance or specification of a variety of cell types[48]. For example, oligodendrocytes are specified in a specific domain of the spinal cord, from which motoneurons also arise. The Appel laboratory has demonstrated that Notch is required for specification of oligodendrocyte progenitor cells (OPCs) within the spinal cord, as constitutively expressed Notch results in an excess formation of OPCs, at the expense of motoneurons[49]. *mind bomb* (*mib*) mutants carry a mutation in the ubiquitin ligase responsible for the internalization of Notch ligand, and thus have a drastic reduction in Notch signaling. This mutation results in excessive neurogenesis[50], and in regard to the epithalamus, these *mib* embryos show increased *leftover* expression and decreased *right on* expression in the right habenula at 56 hours post-fertilization[47] (**Figure 2j**). Thus, as witnessed in other cell types and regions of the CNS, Notch may be crucial to regulate asymmetric neurogenesis in the habenulae.

In order to begin to elucidate the molecular basis of habenular asymmetry, a chemical mutagenesis screen was performed in the Halpern laboratory, with a focus on mutations that result in altered *leftover* expression. This screen produced a mutant with symmetric *lov* expression; *big time* (*bti*) mutants have increased *lov* expression in the right habenula, such that the paired habenulae appear nearly symmetric (**Figure 2k**). The *big time* mutation was mapped to a premature stop codon within the 5th transmembrane domain of the major subunit of the vertebrate translocon, *sec61α1*. This secretory protein is localized on the endoplasmic reticulum and represents the entry point for recently translated or co-translated peptides. We have thus implicated a secretory protein in the regulation of habenular neurogenesis: one possibility is that the translocon mediates habenular asymmetry by a specific regulation of neurogenic molecular components, such as Notch receptors or ligands. Of particular interest are the effects of this mutation on targeting of the interpeduncular nucleus. With expanded *lov* expression in the right habenula, we hypothesize that both habenula now project to both dorsal and ventral regions of the IPN. Further studies investigate the role of this gene in the asymmetric development of the habenula and potential implications for connectivity.

CONCLUSIONS

Asymmetry is a common adaptation of the vertebrate brain, and is assumed to be advantageous as lateralized functions exert a unique pressure on the survival of a species. For instance, a lateralized motor response at a population level could be disadvantageous because predators would learn to predict a given response to stimuli, yet in social populations, such as zebrafish, this disadvantage could be overcome as exploration occurs in pairs or groups[51]. The habenula are an excellent model for asymmetry in organisms such as the zebrafish, which also demonstrates conservation of efferent pathway to the interpeduncular nucleus (from the MHb in mammals), as well as input from the entopeduncular nucleus. This species allows genetic characterization of this asymmetric development, of which the genes are highly conserved in mammals. It is clear that the parapineal has profound influence on the left habenula, yet what signal is it providing? Is Notch signaling necessary to maintain the right habenular neural precursors in an undifferentiated state? Further characterization of habenular neurogenesis, and the genetics that contribute to asymmetric specification and development will begin to elucidate these issues and shed light on this unique, lateralized locus of the dorsal diencephalon.

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FURTHER INFORMATION

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A Novel Pathophysiological Gene-Environment Interaction Suppresses the Neurotoxic Activities of (Mn) and Mutant *HD*

Gunnar F. Kwakye and Aaron B. Bowman§*

Huntington's disease (HD) is a progressive autosomal dominant neurodegenerative disorder characterized by motor impairment, cognitive deterioration, emotional disturbance, and psychiatric disorder, which were first described by George Huntington in1872. Prevalence of HD is approximately 5 in 100,000 worldwide and 1 in 10,000 in the United States with a median age of onset at 39[1]. Surprisingly, approximately 5% of patients have a juvenile form of the disease characterized by increased repeat length in comparison with adult HD onset. Unfortunately, there is currently no effective treatment for the disease. HD is caused by an expansion of a glutamine-encoding triplet repeat (CAG) in the *huntingtin* gene (IT15)[1,2,3]. Interestingly, both genetic and environmental factors have been reported to contribute to variability in age of disease onset. In fact, monozygotic twin[4,5,6,7] and Venezuelan kindred studies[8] have revealed significant environmental influences on the age of onset and clinical presentation of HD. The neuropathology of HD is characterized by selective degeneration of medium spiny neurons (MSNs) in the corpus striatum. The clinical presentation of HD reflects this selective vulnerability with patients exhibiting choreatic hyperkinetic movements. However, as the disease progresses, subsets of neurons within the cortex are lost, whereas the brainstem, cerebellum and hippocampus remain unaffected[1]. Several potential cellular mechanisms of HD neurotoxicity are supported by experimental evidence including alterations in iron homeostasis, energy metabolism, transcriptional regulation, brain-derived neurotophic factor (BDNF) signaling, axonal transport and altered calcium signaling[9-14]. A significant challenge in HD neuropathology is segregating the cellular pathologies into direct and indirect effects of HD neurotoxicity. This review will provide insights into studies that examine how metal ions modulate HD neuropathology. A discussion on wild-type huntingtin protein function and its relation to HD will commence the review, followed by environmental factors and aggregates in HD. The review will conclude with metal ion (manganese) essentiality and how its transport mechanisms may play a role in HD pathology.

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HUNTINGTIN PROTEIN AND HD NEUROPATHOLOGY

The *huntingtin* gene encodes a large 350kDa protein called huntingtin, which when mutated in HD, causes progressive degeneration of the MSNs in the striatum. Huntingtin is a soluble cytoplasmic protein of 3,144 amino acids that is ubiquitously expressed in all regions of the brain and peripheral tissues[14]. The protein is enriched in neurons with similar expression patterns for wildtype and mutant huntingtin[1]. Despite its identification more than a decade ago, the function of wild-type huntingtin remains largely unclear. Huntingtin has many potential domains, boundaries and activities of which are not fully understood. One obviously significant portion of the mammalian protein is the polyglutamine (polyQ) region itself, which has been reported to be present in many transcription factors and aberrantly expanded in other disease-causing proteins[16]. Expansion of the polyQ tract alters the conformational state of the mutant protein and modifies fragmentation by proteolytic processing. Thus, the expanded polyQ tract is required for its subsequent aggregation and accumulation into inclusion bodies[17-21]. Although the relationship between polyQ aggregates and neurotoxicity is complex, recent data has demonstrated an inverse correlation between the polyQ protein inclusions neuropathology at the cellular level[17,22-23]. In unaffected individuals, the polyQ stretch in huntingtin begins at the eighteenth amino acid from the 5' N-terminal region and contains up to 34 glutamine residues[3]. Data from Perutz *et al* in1994 showed that this region forms a polar zipper structure and suggested physiological interactions with other transcriptional factors that contain a polyQ region[25]. Bioinformatics analyses

have reported 37 sequential HEAT repeat domains (named after similarity to domains found in the HD protein, Elongation factor 3, PR65/A subunit of protein phosphatase 2A, and TOR) spanning the entire protein, though other functional domains of the HD protein have also been identified[26-30]. The precise biological activity of HD is unknown, but many HEAT repeat containing proteins function as molecular scaffolds for stable interacting partners. While numerous interacting partners have been identified, the significance of these interactions to normal HD function is unclear. Huntingtin is found in multiple cellular compartments but localizes predominantly to the cytoplasm where it is found as both a soluble protein and in association with membranes including a variety of vesicles, organelles, and the plasma membrane. Subcellular localization may be important for HD pathogenesis, as nuclear localization of the mutant HD protein has been associated with increased neurotoxicity and correlates with neuropathology[31,32]. HD has been implicated in various cellular processes including iron metabolism, transcription, intracellular transport and membrane trafficking, axonal transport and mitochondrial function[33,34]. A major gap in our understanding of the disease mechanism is the absence of a known function for wild-type huntingtin. In 1995, three independent studies showed that the *huntingtin* gene is essential for normal embryonic development and neurogenesis: its complete inactivation in huntingtin-knockout mice results in embryonic lethality before day 8 (before gastrulation and the formation of the nervous system)[35-37]. Most intriguingly, the loss of *huntingtin* gene function in the adult brain results in motor dysfunction and a broad neurodegenerative phenotype, but not specifically for the neurons vulnerable to the polyglutamine-expanded disease protein[37-39]. The effects of polyglutamine expansion on HD function are unclear, although the mutant protein can rescue the embryonic lethal phenotype of the null mouse[40]. Evidence from mouse genetics and the dominant inheritance pattern of HD, indicates that HD is caused predominantly by a toxic gain-of-function, although there is also evidence for a dose-dependent neuroprotective loss-of-function. Thus, a pivotal question in HD research is aimed at understanding how mutant huntingtin causes selective neuronal pathology, especially in the MSNs of the striatum and pyramidal neurons in the motor cortex. One possibility is that environmental agents such as neurotoxic metal ions and toxins may modulate HD pathophysiology by promoting aberrant protein-protein interactions with mutant huntingtin to alter normal wild-type huntingtin physiological functions in striatal and cortical neurons. Thus, metal ions may facilitate mutant huntingtin's toxic gain-of-function processes in HD neuropathology.

ENVIRONMENTAL FACTORS IN HD PATHOPHYSIOLOGY

Over a decade after the identification of the HD mutation, there has been conflicting reports linking complete or incomplete penetrance of HD to triplet repeat expansion length. Fortunately, Rubinsztein and other researchers have provided data which shows that triplet repeat expansion at the HD locus beyond 35 glutamine-encoding CAG repeats is sufficient to cause HD, though repeats between 36-40 show incomplete penetrance[41,42]. Although longer repeat length has been associated with earlier onset, repeat length in general account for only 60% of the variability in age of onset[8]. Thus, it is rationale to link both genetic and environmental factors as likely partners in contributing to HD, specifically, environmental factors contributing to the largest share of residual variability[8,43]. Gómez-Esteban and other researchers in the HD field have revealed significant environmental influences on the age of onset and clinical presentation in monozygotic twin studies that have the same number of expanded repeats[45-48]. Unfortunately, the aforementioned monozygotic twin studies failed to reveal the nature of the environmental factors involved. Animal models of HD have provided further support for the influence of environmental factors on HD onset and progression[43,44]. Indeed, Rozengzweig, Bennet and colleagues since the 1960s have studied the effects of environmental enrichment on the neuroanatomy and neurochemistry in wildtype animals that may enhance memory[49]. With these clear indications that environmental factors can influence HD pathophysiology, it is compelling to probe the possible contributing environmental factors and how they modulate HD pathophysiology.

THE ROLE OF METALS IN NEURODEGENERATIVE DISEASES

In the past decade, there has been a growing interest to understand the metabolism of neurotoxic metals and their influence on various neurodegenerative diseases, such as Manganism, Wilson's, Parkinson's, and Alzheimer's diseases. Occupational and environmental exposures to these metals [Manganese (II) (Mn^{2+}) and other metal ions (e.g. Cu^{2+}, Zn^{2+}, Al^{3+}] have been suggested as a possible cause of neurodegenerative diseases. However, less attention has been focused on metals in HD neuropathology. Currently, there is evidence supporting amyloid fibrillogenesis and aggregation of proteins such as prion protein (PrP) and α-synuclein via Mn^{2+} and other metal ions (e.g. Cu, Al, Zn) interactions[50]. These proteins are metal ion binding proteins that interact with divalent metal ions to play a role in their altered conformational state, solubility, and aggregation[51-56]. However, *in vitro* analysis of

prion protein aggregates have shown that Mn can promote aggregation independent of the PrP metal binding site[55]. Uversky has proposed that polyvalent metal ions, such as Mn, may promote aggregation by cross-linking protein carboxylates[56]. Comparatively, Perutz and Green have also hypothesized that the mechanisms of neuronal intranuclear inclusions (NIIs) of mutant huntingtin (aggregates) in HD is either via polar zipper formation or covalent bonding by transglutaminase-catalyzed cross-linking[24,57]. Given the strong association between protein aggregation, metal ions and neurodegeneration, it is highly rationale to speculate that metal ions have the potential to modulate HD pathophysiology.

RELATIONSHIP BETWEEN ALTERED METAL ION HOMEOSTASIS, METAL TOXICITY, AND HD

The clinical progression of HD has been reported to be associated with altered metal ion homeostasis, wherein iron and copper are significantly elevated in the corpus striatum[58]. In addition, data from animal models have also shown that there is significant increase in the levels of microglia ferritin, an intracellular iron storage protein [59]. In fact, a common phenomenon in multiple neurodegenerative diseases is the alteration of various metal ion levels, and their obvious neurotoxic consequences. Although the distribution of metals throughout the brain is not uniform, metal ion accumulation in specific brain regions reflects neurotoxicity (example: manganese accumulation and neurotoxicity in the globus pallidus results in manganism). Interestingly, Fox *et al* have recently reported that huntingtin protein interacts with Cu ions, with this specific metal binding decreasing the solubility of wild-type huntingtin protein[60]. However, the cellular effects of Cu or other metal ions on HD function, proteolytic processing to generate N-terminal fragments, aggregation of fragments, and formation of mutant huntingtin inclusion bodies remain unknown. A recent study suggested that inclusion bodies formed by CAG expansion in mutant huntingtin protein fragments are associated with iron-dependent oxidative events, opening the possibility that other redox-reactive metal ions, such as Mn, may influence polyglutamine aggregation[61]. In essence, several studies have provided evidence that supports a role for oxidative stress, mitochondrial dysfunction, excitotoxicity, and alterations in iron homeostasis as critical steps in both Mn neurotoxicity and HD neuropathology. Importantly, chronic exposure of Mn in animal models shows significant accumulation in the striatum, providing any potential interaction between Mn and HD to occur within the neurons most vulnerable to HD pathology. Unfortunately, there are currently no reported studies examining the connection between metal exposure, including Mn and HD neuropathology. With the increasing evidence supporting strong association between metal ions and protein aggregation, similarities between metal ion cytotoxicity and cellular pathways of neurodegeneration, altered metal ion homeostasis, and the differential accumulation of various metals across neuronal subtypes, it is highly rationale to propose that metal ions with neurotoxic properties are the strongest candidates for the largest residual environmental variability that has been hypothesized to modulate selective neurodegenerative process in HD. In an attempt to identify the link between HD and metal ions, our lab has screened the impact of several neurotoxic metal ions on a striatal cell line model of HD and found striking interactions between mutant huntingtin expression and Mn exposure, wherein mutant huntingtin protein and Mn suppress the neurotoxic activities of each other. The remaining sections of this review will focus on manganese essentiality, neurotoxicity, mechanisms of transport and its possible link to HD.

MANGANESE: ESSENTIALITY AND NEUROTOXICITY

Mn is an essential ubiquitous trace element required for normal growth, development and functioning in all bodily tissues, and cellular homeostasis[62]. In humans and animals, manganese functions as a cofactor for several Mn-dependent enzymes that are appropriate for neuron or glial cell function, as well as enzymes involved in neurotransmitter synthesis and metabolism. These Mn-dependent enzymes include glutamine synthetase, pyruvate decarboxylase, superoxide dismutase 2 (SOD2), and arginase[63]. The idea of Mn involvement in HD stems from earlier studies by Butterworth in 1986 where it was shown that there are significant decreases in Mn-dependent enzymes, specifically, glutamine synthetase and pyruvate carboxylase in the caudate nucleus of HD patients[64]. Interestingly, given Mn essentiality, inadequate intake of Mn can result in abnormal glucose tolerance[65]. Despite its essentiality in multiple metabolic functions, Mn can be toxic at high concentrations. The brain in particular is highly susceptible to Mn neurotoxicity. Excessive dietary intake and environmental exposures to Mn for longer periods result in accumulation of Mn in the globus pallidus, striatum and subthalamic nucleus of the basal ganglia, which causes a clinical disorder referred to as manganism. This disorder causes extrapyramidal symptoms that resemble idiopathic Parkinson's disease (IPD). Although extensive studies have been conducted to link altered manganese levels to IPD, the connection between manganese and HD remain unknown. Mn exposure and increased brain Mn levels may modulate other neurodegenerative

diseases (example: Alzheimer's and polyglutamine diseases) in which protein aggregation and amyloid deposition are parts of the pathophysiology. Recent data shows that non-human primates exposed to Mn have diffuse amyloid-Beta plaques in the frontal cortex, similar to what is seen in Alzheimer's patients[66]. This is particularly interesting in light of the observation that Mn levels are elevated in Alzheimer disease brains[67]. Indeed alterations in various brain metals, including Mn, have been suggested to modulate sensitivity to oxidative stress, which likely plays a fundamental role in the pathophysiology of most neurodegenerative disease states. For example, a possible mechanism by which Mn exposure may modulate neurodegenerative conditions is through alterations in Mn-dependent antioxidant enzyme SOD2 level or activity. It is known that reduction of SOD2 levels enhances Alzheimer's disease pathology in a transgenic mouse model[68]. Preferentially enhanced NMDA (N-methyl-D-aspartic acid) receptor mediated excitotoxicity, mitochondrial dysfunction, and oxidative stress have also been implicated in HD[1,69]. Exposure of rats to Mn was found to decrease the levels of two manganese bound enzymes, SOD2 and glutamine synthetase, in the basal ganglia[70]. Furthermore, recent data from HD mouse models have also linked altered arginase activity resulting in urea cycle deficiency[71]. Thus, Mn exposure may diminish the activities of Mn-dependent enzymes, thereby contributing to the HD pathophysiology.

MECHANISMS OF MANGANESE TRANSPORT

Unpublished data from our lab shows that mutant HD striatal cell cultures are resistant to Mn toxicity relative to wild-type, over a broad Mn concentration range. This observation suggests a strong interaction between mutant huntingtin and Mn. It is highly rationale to speculate perturbations in Mn transport (import and export) and storage in the aforementioned mutant striatal cell line models of HD. The remainder of this review will focus on the mechanisms of manganese transport. Due to the delicate relationship between Mn's essentiality and toxicity, both the absorption and tissue levels of this metal are tightly regulated. Mn can cross the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCB) on several carrier(s) and in different oxidation states[72,73]. Unfortunately, no unique mammalian Mn transporter has yet been identified. It appears that several potential modes of Mn transport across the BBB and BCB have been reported and others speculated to occur via facilitated diffusion, active transport, divalent metal transporter 1 (DMT-1)-mediated transport, voltage-regulated and stored operated Ca^{2+} channels, ZIP8, citrate, and transferrin (Tf)-dependent transporters[72]. Of all the above listed polyvalent transporters, Tf and DMT1 are the most extensively documented[74]. Approximately 80% of Mn in plasma is bound to $beta_1$-globulin and albumin and a smaller fraction of Mn is bound to transferrin (Tf), an iron-binding protein[75]. Mn binding to Tf is time-dependent and Tf receptors have been shown to be present on cerebral capillary surfaces[74]. When complexed with Tf for transport across the BBB, Mn is exclusively present in the trivalent oxidation-state[76]. Another critical regulator of brain Mn levels is the divalent metal transporter (DMT-1). The transporter belongs to the family of natural resistance-associated macrophage protein (NRAMP) and has also been referred to as the divalent cation transporter (DCT). Gene transcription of this protein is regulated by Fe concentration via a Fe-response element (IRE) located on the mRNA[77]. Orthologous mutations (glycine 185 to arginine) in the DMT-1 gene of the Belgrade (b) rat, and microcytic anemia (mk) mouse result in significantly lower Mn and Fe tissue levels, including the brain[78,79]. The reduction in brain Mn and Fe uptake in the above animal models suggests that defective DMT-1 allele alters the disposition of both metals and that Mn and Fe may utilize DMT-1 as a putative transporter across the BBB and BCB. These results support the notion that impaired DMT1 alters Mn transport due to a defect in Mn uptake, export and storage.

Given the above evidence of metal ions (example, Mn) essentiality and neurotoxicity in neurodegenerative diseases, specifically HD, and alterations in Mn transport and levels in HD animal models, it is highly likely that there exists a gene-environment interaction between mutant huntingtin and Mn that may modulate HD pathophysiology. However, the identification and functional characterization of both Mn transport pathways and Mn-bound proteins is not completely understood. Thus, further investigations would have to be conducted to support the gene-environment interaction hypothesis between mutant huntingtin and Mn in HD neuropathology.

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FURTHER INFORMATION

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Genetic Influences on Neural Circuitry for Human Reward Processing

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From the drunkard Noah of the Old Testament to the cannabis abusing hashishins of 12th century Persia and on to the present day, drug and alcohol addiction have been recognized as a scourge of mankind since the beginning of recorded history. Current estimates suggest that as many as 9% of Americans meet the DSM-IV criteria for substance use disorders[1,2], and the economic burden of substance abuse (including costs relating to crime, lost productivity, treatment, incarceration and law enforcement) has been assessed at approximately half a trillion dollars[3]. Thus, addiction is a highly prevalent and enormously costly public health issue. However, it is noteworthy that despite the fact that all drugs of abuse are highly reinforcing, only a relatively small percentage of individuals exposed to these drugs go on to develop the destructive pattern of compulsive drug seeking and use that is the hallmark of addiction[4]. Characterizing sources of individual differences in risk and elucidating their mechanisms of action will aid in the identification of novel therapeutic targets for addiction; as such, these research aims represent crucial next steps in advancing treatment options for individuals afflicted with substance use disorders.

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Family, adoption and twin studies have demonstrated that heritable influences account for a moderate-high proportion of population variance in risk for addiction, and therefore suggest that genetic mechanisms may predispose susceptibility[5-7]. In general, when attempting to identify etiopathophysiological pathways through which heritable factors might exert their effects on susceptibility for a given disorder, it is instructive to consider the core cognitive and behavioral domains that are disrupted in that disorder[8]. Addiction is fundamentally a disease of reward and motivation, and it is commonly accepted that addiction develops through the arrogation of evolutionarily conserved neural systems for processing survival-critical natural rewards (e.g. palatable food, sex) by drugs of abuse[9-13]. This singular fact raises the intriguing possibility that genetic risk factors may shape susceptibility by altering the functional properties of brain reward circuitry. The use of functional neuroimaging to characterize the impact of genetic variation on brain structure, function and connectivity is one experimental approach that offers the promise of confirming this hypothesis[8]. However, such an approach must be guided by a tenable conceptual model of reward, and girded by a comprehensive understanding of the genetic, pharmacological, anatomical, and functional architectures of brain reward systems. In what follows, we will outline a current influential conceptualization of reward; review the neurochemistry of "classic" mesolimbic and mesocortical dopaminergic reward circuitry; discuss the relationship between dopamine signaling and dissociable aspects of reward processing; detail findings from human functional imaging studies using reward paradigms; and present recent data implicating genetic variation in dopamine signaling as a source of individual differences in reward response.

A TRIPARTITE MODEL OF REWARD: LEARNING, MOTIVATION AND HEDONICS

A barely noticed television commercial cues a desire for ice cream. Anticipating the impending delights of a chocolate cone, you drive to Ben and Jerry's to obtain the desired treat. Consumption of the cone produces a subjective sense of pleasure. A moment's reflection on even the simplest of reward episodes reveals that reward is not a unitary construct, but rather comprised of several discrete constituent processes. Berridge and Robinson have outlined three basic psychological components: learning, motivation and affect[14]. Generally speaking, reward learning involves ascertaining predictive relationships among external stimuli, interoceptive sensations, and actions. For example, in a simple form of associative reward learning—pavlovian appetitive conditioning—reward-predicting conditioned stimuli (reward cues) energize behavioral responses appropriate to the facilitation of reward consumption. Reward learning mechanisms operate interactively and in parallel with neural systems involved in ascribing hedonic and motivational value to stimuli. These systems underpin

the ability of a rewarding stimulus to induce a positively valenced affective state (pleasure) and elicit a motivational drive that prioritizes future (re)attainment of that state and organizes goal-directed behavior towards this end (desire). While these two reward components usually co-occur and are thus often experimentally conflated, Berridge and Robinson were among the first to argue in favor of a clear differentiation of these facets, which they term 'liking' and 'wanting,' respectively[15]. 'Liking' refers to the hedonic impact of a stimulus—the positively valenced sensory experience that immediately follows reward receipt. By contrast, 'wanting' or 'incentive salience' refers to the motivational value of that reward—that is, its ability to drive goal-directed behavior. The separation between 'wanting' and 'liking' echoes the distinction, first made by ethologists in the late 19th/early 20th century, between "appetitive" and "consummatory" phases of reward behavior. According to this classification scheme, goal-directed approach behavior aimed at obtaining a reward is considered to be part of the 'appetitive phase,' while consumptive (food reward) or copulative (sex reward) behaviors initiated upon reward receipt were considered part of the "consummatory" phase. Neurobiological discrimination of "liking" and "wanting" processes arose from the finding that experimental manipulation of the neurotransmitter dopamine (DA) appears to have a dissociable impact on behavioral measures of each. Namely, altering mesolimbic dopamine signaling has a specific and profound effect on reward 'wanting,' while reward 'liking' is unaltered by such changes[14]. Berridge and Robinson have hypothesized that dysregulation within mesolimbic dopamine circuitry for reward 'wanting' following exposure to drugs of abuse underlies compulsive drug seeking and drug taking behaviors in addiction. Prior to discussing these findings, I will review relevant anatomical and pharmacological aspects of dopaminergic neurotransmission.

DOPAMINE: ANATOMY AND PHARMACOLOGY

Dopaminergic cell bodies are localized to several discrete mesencephalic nuclei; forebrain innervation arises from two of these: the substantia nigra pars compacta (SN) and the ventral tegmental area (VTA). Ascending dopamine axons project via the median forebrain bundle (MFB) to form three relatively circumscribed pathways. The nigrostriatal system projects from SN to dorsal striatum (caudate and putamen); this system is involved in motor control, executive function and habit learning. The mesolimbic system originates in VTA and projects to ventral striatum (including nucleus accumbens; NAcc) and other limbic targets, such as amygdala and hippocampus. The mesocortical system emanates from the VTA as well and projects to cortical regions; cingulate, orbitofrontal and medial prefrontal cortices (PFC) receive particularly dense mesocortical innervation. Mesolimbic and mesocortical dopamine circuits are involved in diverse aspects of cognition and behavior, including motivation and associative learning (mesolimbic system; see below) and attention, working memory, and inhibitory control (mesocortical system).

Dopamine is synthesized in presynaptic nerve terminals from the essential amino acid L-tyrosine. Following the conversion of tyrosine to L-DOPA by tyrosine hydroxylase (TH)—the rate-limiting step of dopamine synthesis—L-DOPA is stripped of its carboxyl group by the enzyme amino acid decarboxylase (AADC) to form dopamine. After synthesis, dopamine is packaged into synaptic vesicles within the presynaptic terminal by the vesicular monoamine transporter (VMAT2). Excitatory stimulation of midbrain dopamine neurons causes dopamine release from axon terminal sites. Following release, extracellular dopamine is either cleared from the synaptic space or binds to a G-protein coupled receptor (GPCR) to initiate signal transduction. Clearance is accomplished by reuptake or enzymatic degradation. The presynaptic membrane-bound dopamine transporter (DAT) binds dopamine with high affinity and, under normal conditions, transports released neurotransmitter back into the presynaptic terminal for repackaging into vesicles or enzymatic breakdown. Dopamine is catabolized by monoamine oxidase (MAO) present in axon terminal mitochondria and in glia, and by catechol-o-methyltransferase (COMT), found extrasynaptically and postynaptically[16].

Alternatively, dopamine can bind to one of several GPCR subtypes. Dopamine receptors are classified into two families on the basis of sequence homology: D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, D_4). D_1-like receptors (D_1Rs) are exclusively post-synaptic and are coupled to the G-protein $G_{\alpha s}$; stimulation of D_1Rs activates adenylyl cyclase (AC). D_2-like receptors (D_2Rs), which are located both pre- and post-synaptically, are $G_{\alpha i}$-linked and have an inhibitory effect on AC. Somatodendritic D_2 autoreceptors regulate dopamine nerve cell firing, while stimulation of presynaptic terminal D_2 autoreceptors attenuates dopamine synthesis and release. The downstream effects of postsynaptic dopamine receptor binding are mediated by the activation (by D_1Rs) or inhibition (by D_2Rs) of AC, which in turn influences production of cyclic adenosine monophosphate (cAMP) and thus the function of cAMP dependent protein kinase A (PKA). In the striatum, PKA governs the activity state of DARPP-32 (dopamine-and cyclic AMP-regulated

phosphoprotein with molecular weight 32 kDa), a "master molecular switch" that is known to regulate (by phosphorylation) the activity of a variety of cell-surface receptors and ion channels. In sum, dopaminergic signal transduction is a complex, multi-stage process that is highly regulated at each stage. Inter-individual variability (e.g. due to genetic variation) in the functionality or concentration of proteins involved in any of these stages—dopamine synthesis, vesicular sequestration, release, reuptake, enzymatic degradation, receptor binding or downstream messenger signaling—could be expected to influence individual differences in the functional characteristics of dopaminergic circuits outlined above, and by extension, aspects of cognition, emotion and behavior subserved by them[16].

DOPAMINE, WANTING AND LIKING

Interest in dopamine as a neurochemical substrate for reward developed from research into the neural basis of reinforcement motivation. In their seminal work, Olds and Milner used intracranial electrical self-stimulation to identify brain regions where animals would work for continued electrical stimulation. They found that self-stimulation behavior was most robustly elicited when electrodes were placed in sites along the MFB; Olds termed these sites "pleasure centers[17]." Subsequent work by Roy Wise and others implicated the involvement of SN and VTA dopamine neurons in electrical self-stimulation[18], detailed the sensitivity of MFB stimulation reward to pharmacological intervention with dopaminergic drugs[19], demonstrated that all drugs of abuse increase synaptic dopamine in the NAcc[20], showed that animals will work for the opportunity to self-administer dopamine potentiating drugs[21-23], and appeared to suggest that such drugs reinforce instrumental behavior only to the extent that they elevate dopamine[24]. These and related findings led Wise to develop the hedonia hypothesis of dopamine, which held that "dopamine junctions represent a synaptic way station…where sensory inputs are translated into the hedonic messages we experience as pleasure, euphoria or "yumminess[25]." This hypothesis is the conceptual foundation for many of the dominant neurobiological theories of drug addiction (e.g. the reward allostasis model of Koob and LeMoal[11]), which share the view that addiction is a disorder of meso-accumbens dopamine "pleasure" systems. Wise's formulation of reward neurochemistry was premised on the assumption that the hedonic and motivational values of a stimulus are so inextricably linked as to be indistinguishable. It was presumed that if a food or drug is pleasurable, an animal will work to obtain it, and conversely, that the degree to which an animal works to obtain a reward is in direct proportion to its hedonic value. Thus, for Wise, evidence that dopaminergic manipulations affected drug-seeking and consumption was considered confirmation that dopamine was necessary for producing the hedonic effects presumed to drive such goal-directed behaviors. However, Berridge and colleagues challenged this assumption by using experimental measures that allowed them tease apart hedonic and motivational responses to rewards. Such designs permitted the demonstration of dissociable neural substrates for reward 'wanting' and reward 'liking'.

Utilizing affective facial expressions as an objective and quantifiable measure of hedonic response to gustatory reward stimuli (e.g. sucrose), a range of dopaminergic interventions have been found to have little to no impact on hedonic 'liking' reactions despite profound effects on behavioral indices of motivation. For example, 6-hydroxy-dopamine (6-OHDA) lesions of ascending dopaminergic projections have no effect on hedonic responses to sucrose, despite almost completely depleting dopamine levels in NAcc and dorsal striatum[26-27]. In addition, D_2R blockade does not alter 'liking' responses (to sucrose) or 'disliking' responses (to quinine)[28]. Similarly, neither systemic administration of amphetamine[29], amphetamine microinjections into NAcc[30], or electrical stimulation of the MFB[31] affect liking reactions to sucrose reward, although all three of these manipulations significantly potentiate manifestations of reward 'wanting,' such as food seeking and ingestive behaviors. Notably, genetically hyperdopaminergic and hypodopaminergic mice (DAT and TH knockouts, respectively) show striking and directionally consistent alterations in reward 'wanting' behavior (DAT knockouts increased, TH knockouts decreased) in the absence of corresponding changes in hedonic response[32-36]. In aggregate, these findings strongly suggests dissociable neural mechanisms for ascribing motivational and hedonic value to rewards, with dopamine selectively mediating reward 'wanting' but not reward 'liking'. Berridge and Robinson's Incentive Salience model and Incentive Sensitization hypothesis developed directly from these observations.

INCENTIVE SALIENCE AND INCENTIVE SENSITIZATION

Based on the findings outlined above, Berridge and Robinson have argued that mesolimbic dopamine mediates the dynamic attribution of "incentive salience." This value, when ascribed to a reinforcing stimulus, "transforms mere sensory information about rewards and their cues…into attractive, desired, riveting incentives…to make [them] a 'wanted' target of motivation[14]." Incentive salience "tags" a stimulus as a target for goal-directed behavior and ensures that

an organism will prioritize resources towards obtaining that stimulus over others. Noting that that the key neurobiological nexus for the actions of drugs of abuse—meso-accumbens dopamine circuitry—is critically involved in ascribing incentive salience to environmental stimuli, Berridge and Robinson have hypothesized that drug addiction involves a dysregulation of incentive salience processing. Their "Incentive Sensitization" hypothesis is based on the observation that drugs of abuse induce a profound and long-term hypersensitivity of this system to rewards and to reward-predicting cues. Repeated administration of a wide range of addictive drugs causes animals to become sensitized to their psychomotor effects (e.g. elevated locomotor, exploratory and approach behavior). Strikingly, repeated exposure to psychoactive drugs induces sensitization to their incentive motivational effects, even as tolerance develops to their hedonic effects. For example, pre-exposure to amphetamine decreases the dose and the time required for an animal to subsequently learn to self-administer the drug, and increases the amount of work they will expend to gain access to it[23,37-38]. The expression of sensitization is strongly influenced by associative learning mechanisms, with drug associated cues promoting excessive 'wanting' behavior long after the last drug exposure[39]. The development of sensitization is paralleled by structural adaptations in NAcc dendritic spines, and by cellular alterations within the VTA and at NAcc/PFC synapses[40-42]. In sum, the Incentive Sensitization hypothesis posits that repeated exposure to an addictive drug sensitizes meso-accumbens circuitry for incentive motivation, leading to an excessive attribution of incentive salience to the drug and to drug-related stimuli, even in the face of diminished hedonic responses to the drug over time. In this way, meso-accumbens sensitization by drugs of abuse causes addicted individuals to 'want' the drug more and more, engaging in increasingly compulsive and destructive behaviors to obtain these drugs, even as they may come to 'like' the drugs less and less.

INCENTIVE SALIENCE AND THE HUMAN NAcc: FUNCTIONAL IMAGING STUDIES

Human functional neuroimaging studies recapitulate the distinction between wanting and liking by elucidating distinct neuroanatomical substrates for each, and suggest that reward-related NAcc activity in humans is specific to incentive salience. Several early fMRI studies demonstrated that monetary reward and drugs of abuse robustly activate mesolimbic and mesocortical dopamine terminal fields in humans[43-47]. In addition, monkey electrophysiological work by Schultz revealed differences in the response patterns of NAcc and orbitofrontal neurons to the expectation and delivery of rewards, suggesting a neuroanatomical basis for the distinction between appetitive and consummatory phases of reward recognized by ethologists[48]. Drawing on this body of work, as well as its conceptual links to Berridge and Robinson's incentive salience model of reward, Knutson and colleagues have found that anticipating and receiving monetary rewards activate distinct neural circuits. NAcc is active following the presentation of cues that signal the opportunity to emit an instrumental response to obtain reward, but not during the receipt of that reward; by contrast, medial prefrontal cortex is active following the attainment of monetary reward, but not during the anticipatory period preceding reward receipt[49-52]. Similar results have been observed during the anticipation and receipt of taste reward[53]. Further support for the notion that human NAcc is sensitive to the motivational aspects of reward, rather than reward hedonics, is offered by data showing that NAcc response to monetary reward is contingent on stimulus saliency[54] and dependent on the production of an instrumental response[55-56]. Finally, NAcc activity is associated with cue-induced craving (wanting) in abstinent substance abusers[57-59], and a recent fMRI study found that NAcc activation following acute cocaine administration was positively correlated with subjective ratings of drug craving, but negatively correlated with subjective ratings of drug "high" (liking)[60]. These findings imply a specific and circumscribed role for NAcc in human reward processing: the attribution of incentive salience ('wanting') to reinforcing stimuli.

INCENTIVE SALIENCE AND THE HUMAN NAcc: BEHAVIORAL PHARMACOLOGY AND RECEPTOR IMAGING

fMRI signal is dependent on task-driven hemodynamic changes that are correlated with changes in local field potentials; as such, it is thus a fundamentally indirect measure of brain activity[61]. In addition, while preclinical research is increasingly supportive of the notion that NAcc fMRI reward signal is driven by dopamine signaling[62], this has yet to be definitively confirmed. Therefore, a series of behavioral pharmacology and radioligand PET studies provide a critical complement to the fMRI work outlined above by demonstrating that dopaminergic activity in the NAcc is necessary and sufficient for human reward wanting. Using a dietary manipulation that acutely depletes catecholamine levels (acute catecholamine depletion; ACD), Leyton and colleagues demonstrated that ACD significantly attenuates stimulated dopamine release in the NAcc[63], selectively decreases subjective "wanting" ratings following intranasal cocaine without affecting ratings of cocaine-induced pleasure[64], and impairs motivated

responding to reward predicting cues without altering hedonic responses to amphetamine[65]. This same group found that the magnitude of amphetamine induced dopamine release in the NAcc is strongly correlated with self-reported 'drug wanting'—and with individual differences in "novelty seeking" trait scores—but not with amphetamine-linked changes in positive affect[66]. Similarly, elevated stimulated NAcc dopamine release has been linked to compulsive drug wanting, but not drug liking, in patients with Parkinsons disease who abuse L-DOPA[67]. In the gustatory domain, methylphenidate-induced striatal dopamine release increases non-hedonic ratings of appetitive motivation for food[68]. Of note, it has been shown that amphetamine-associated conditioned cues increase NAcc dopamine release to an extent that is comparable to the drug itself[69], mirroring fMRI data (vide supra) that implicate NAcc in cue-induced craving. Furthermore, building on the results of prior behavioral experiments [70-72], Boileau and colleagues have established a relationship between stimulant-induced sensitization and NAcc dopamine in humans. They administered a constant dose of amphetamine to participants on three occasions; the second and third exposures were 14 and 365 days after the first exposure, respectively. Relative to first exposure, they found that psychomotor responses and amphetamine-induced dopamine release in NAcc were markedly potentiated on the second and third exposures. Remarkably, the magnitude of sensitized response was strongly correlated with individual differences in "novelty seeking" trait scores and self-report impulsivity measures related to addiction risk[73]. Taken together, these data suggest that NAcc dopamine function is associated with incentive salience, mediates a conditioned 'wanting' response, and is sensitized by exposure to drugs of abuse—all of which are predicted by the Incentive Sensitization hypothesis of addiction.

GENETIC VARIATION IN MESOLIMBIC DA SIGNALING AS A RISK FACTOR FOR ADDICTION

As outlined above, converging evidence identifies NAcc dopamine signaling as a core neurobiological substrate for reward 'wanting,' a reward component process that is putatively dysfunctional in addiction. Supporting a role for NAcc DA in addiction, substance abusers consistently show alterations in mesolimbic DA function, including decreased NAcc D2R availability[74-76] and increased NAcc fMRI activation to drug cues[77-79]. Further, the personality traits predicted by individual differences in mesolimbic DA function—novelty seeking, sensation seeking and impulsive temperament—are strongly linked to substance abuse risk[66,73,80-84]. Considering the high genetic liability to addiction, these findings imply that some of the variance in addiction risk may be explained by heritable individual variation in DA function. It is thus worth noting that polymorphic markers in dopamine signaling pathway genes have been associated with both addiction-linked temperament factors and to substance abuse diagnosis. Specifically, allelic variants in genes encoding MAOA, COMT, DAT, TH, AADC, VMAT2, and dopamine receptor subtypes 1-5 have been linked to high novelty seeking and impulsivity and to drug and alcohol addiction[85-108].

The relationship between addiction, reward 'wanting,' and mesolimbic DA suggests that risk-variants in dopaminergic genes may influence the development of addiction by affecting the sensitivity of meso-accumbens 'wanting' circuitry to reward-related stimuli. Data from several recent "imaging genetic" studies appear to confirm this hypothesis by linking such variants to individual differences in the NAcc response to reward. Forbes and colleagues examined the impact of four common functional polymorphisms in the COMT, SLC6A3 (DAT1), DRD4 and DRD2 genes on reward-related brain activity: a variable number tandem repeat (VNTR) polymorphism in the 3' region of the DAT1 gene, a non-synonymous (val158met) coding single nucleotide polymorphism (SNP) in exon 4 of the COMT gene, an insertion/deletion (ins/del) polymorphism in the 5' promoter region of the DRD2 gene, and a VNTR in exon four of the DRD4 gene. These variants have been linked to elevated synaptic dopamine and attenuated postsynaptic inhibition via decreased DA clearance (DAT1 and COMT)[109-111], reduced receptor expression (DRD2 and DRD4)[112-113] and diminished agonist-stimulated signaling (DRD4)[114-115]. Carriers of alleles in DAT, DRD2 and DRD4 associated with increased striatal DA release, increased synaptic DA availability, and decreased postsynaptic inhibition exhibited significantly larger NAcc responses to monetary reward[116]. Further, the magnitude of NAcc response positively predicted impulsive temperament, an important risk factor for substance abuse[117-119]. Of note, the same DRD4 allele (the 7-repeat allele) associated with increased NAcc sensitivity to monetary reward is enriched in substance abusing individuals[88,120-121] and DRD4 7-repeat carriers show exaggerated NAcc engagement to alcohol-associated cues. Moreover, the magnitude of increased NAcc response as a function of DRD4 genotype predicts self-report measures of alcohol use, such as frequency and amount[122].

Despite positive findings for variants in DAT1, DRD2 and DRD4, Forbes and colleagues found no effect of the COMT val158met polymorphism on NAcc reward-related activity. However, the task design in that study conflated reward anticipation and reward feedback—an important behavioral distinction

with clear implications for NAcc reward function, as outlined above. Using tasks designed to isolate brain activity associated with reward anticipation[50], two studies have found that COMT genotype is significantly associated with NAcc activity[123-124]. In both studies, the low-activity 158Met allele, linked to increased DA availability and overtransmitted in alcoholism[96,125-126], predicts increased NAcc response to the anticipation of monetary reward. The discordance between these findings and those of Forbes and colleagues suggests that the manifestation of genetic effects on NAcc function critically depends on task characteristics. It remains to be seen if the impact of other DA genetic variants on NAcc reward-related activity is specific to reward anticipation/'wanting'. Of note, allelic variants in downstream dopamine signaling elements, including PPP1R1B (DARPP-32), RGS4, and AKT1, have also been shown to affect striatal structure, frontostriatal connectivity and striatal activity in non-reward paradigms[127-129]. On the whole, these findings imply that addiction-associated genetic variation at multiple nodes within the DA signaling pathway converges to increase the sensitivity of mesolimbic DA circuitry to rewarding stimuli. That these genetic influences on NAcc function are related to clinically relevant behavioral phenotypes (such as impulsive temperament and alcohol use frequency) strengthens the notion that genetically mediated NAcc hypersensitivity may be an important aspect of the neurobiological risk architecture of addiction.

CONCLUSIONS

Herein, we have detailed findings that identify mesolimbic dopamine signaling as a core neurobiological mediator of incentive salience or reward 'wanting', a psychobehavioral process that may be disrupted in addiction. Preliminary functional imaging evidence indicates that heritable variation in dopamine pathway genes may regulate the sensitivity of mesolimbic DA circuitry to rewarding stimuli. Risk-associated genetic variants may exert their deleterious effects by sensitizing NAcc response to such stimuli, perhaps resulting in the hyperattribution of incentive salience in genetically susceptible individuals following exposure to drugs of abuse. In addition, genetically influenced alterations in mesolimbic DA signaling may hasten the development of incentive sensitization by reducing the number drug exposures required to induce sensitization of drug seeking and consumptive behavior. Such changes could lead to an acceleration of the process by which drug use behaviors shift from "recreational" to "compulsive." Future imaging studies might endeavor to examine the impact of known functional variants on specific aspects of reward processing, particularly reward anticipation/'wanting', and on the neural correlates of psychostimulant sensitization (cf. Boileau *et al*). In addition, using individual differences in NAcc reward response or amphetamine-sensitized stimulated DA release as a quantitative trait, novel susceptibility alleles could potentially be identified by genome-wide screens, a strategy that has yielded significant findings in other cognitive domains (e.g. memory[130]). A combination of top-down (neuroimaging phenotype to genotype) and bottom-up (genotype to neuroimaging phenotype) approaches is one promising investigative strategy for finding new pathophysiological pathways in addiction; one or more of these may prove amenable to therapeutic intervention.

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FURTHER INFORMATION

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Perceptual-Training Induced Narrowing of the Multisensory Binding Window

Albert R. Powers III and Mark T. Wallace[§]*

While it has long been understood that accurate perception of events in the environment requires the successful combination of information from all senses, researchers have only recently begun to uncover the powerful perceptual and behavioral benefits arising from this combination. The study of how multisensory information shapes our view of the world around us has exploded in recent years (see [1-2] for reviews), and current investigation has begun to focus upon the neural substrates underlying these multisensory interactions.

INTRODUCTION: MULTISENSORY INTERACTIONS

Examples of multisensory interactions fill nearly every aspect of our lives. One common everyday example is the increase in speech intelligibility experienced when a speaker is visible[3]. Psychophysical research involving human subjects has provided numerous other examples of how multisensory interactions influence perception and behavior. The most basic of these include the speeding of responses[4-6] and the improved detection of targets when information from two sensory modalities is presented[7-9]. The interactions behind these two examples clearly confer an adaptive benefit. Multisensory illusions, although unlikely to have such benefits, further illustrate the power of multisensory interactions to shape our perceptions and behaviors in the absence of our conscious knowledge. In the Flash-Beep Illusion[10-11], participants frequently perceive multiple flashes of light when two sounds are presented, even when only a single flash actually occurred. In the ventriloquist effect, perception of the location of a sound source can be shifted by the presence of a temporally coincident but spatially disparate visual cue[12-14]. In the realm of speech, the McGurk Effect uses simultaneous presentation of visual /ga/ and auditory /ba/ to produce a fused percept that reflects a synthesis of the visual and auditory channels (/da/ or /tha/)[15-16]. These multisensory interactions are not unique to the audiovisual realm. One of the more entertaining multisensory illusions, for example, is the somewhat alarming "parchment skin illusion" wherein changing the frequency of the sound of one's fingers rubbing together alters the tactile perception of that action from "like rubbing against glass" to "like rubbing against sandpaper"[17-18]. Many other tasks of daily life are inherently multisensory in nature, from tasting food to reading. Purposeful manipulation of the processes underlying multisensory interactions, then, carries potential to alter our most basic experiences in very profound ways.

PRINCIPLES OF MULTISENSORY INTEGRATION AND THE TEMPORAL BINDING WINDOW

Conventional knowledge of multisensory integration in both humans and animal models indicates that multisensory interactions are guided by a set of principles that ultimately relate to the nature of the stimuli that are being that multisensory neurons (*i.e.*, those neurons that respond to or are influenced by multiple sensory modalities) are likely to show the largest multimodal response gains when the stimuli presented are spatially proximate[19-20]. The second is the rule of inverse effectiveness, stating that the largest gains are seen when stimuli that are only weakly effective on their own are paired[21]. Most germane to the current work, the temporal principle posits that close temporal pairing of multisensory stimuli results in the most significantly enhanced behavioral or electrophysiological responses[22]. Instances of this rule's application in perception and behavior abound[23-25], and examples of its validity in non-invasive human electrophysiology are also plentiful[26-28]. Although these examples indicate that the greatest response gains are seen when there is a close temporal relationship between stimuli of different sensory modalities, there appears to be a window of time within which the pairing of multisensory stimuli results in a significantly enhanced behavioral or electrophysiological response.

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We refer to this interval in a general sense as the temporal window of multisensory integration (**Figure 1**).

Several studies have focused upon this concept of a multisensory temporal binding window and have begun to define its boundaries in human behavioral studies[25,29-34]. The boundaries of the temporal window of multisensory integration can be delineated psychophysically by identifying the range of audiovisual asynchronies over which a multisensory interaction (e.g., a change in performance or perception) is observed. Dixon and Spitz[35] first defined the window in just this way, and their findings have been replicated on other psychophysical tasks[36-37]. However, though the window's boundaries have been well established using several different psychophysical tasks, the literature have surprisingly little to say about the permanence of these boundaries and their ability to be manipulated in time.

SENSORY PLASTICITY AND THE TEMPORAL WINDOW

The brain's ability to alter its structure and function based upon input from the environment ranks among its most evolutionarily valuable traits. Seminal early developmental studies showed that this plasticity can be driven in a bottom-up fashion by exposure to a constrained set of sensory stimuli[38-40] and that passive exposure to these stimuli becomes less likely to drive behavioral change and neural reorganization as an animal reaches the end of a critical period of development[41]. Later, electrophysiological studies revealed that both the behavioral and anatomical changes typically elicited in developing animals by passive exposure can indeed take place in adults via top-down perceptual training, wherein stimuli are paired with either reward or punishment[42-44].

In humans, perceptual training studies have highlighted the ability of the individual sensory systems to exhibit plastic change. For example, it has been demonstrated that adults with amblyopia exhibit improvement in Vernieracuity judgments following training[45-46], and in the auditory realm, that adults demonstrate accuracy gains on synchronicity judgments and temporal order judgment tasks following practice[47-48]. In these studies, while subjects showed improvement in the task on which they were trained, training effects did not generalize to a separate, albeit related, task.

Indeed, lack of transfer between tasks in perceptual training paradigms is common[49-50], especially in perceptual training studies focusing upon a unimodal task. The extent to which perceptual training generalizes across stimuli[51] and across tasks[48] has been hypothesized to vary according to the level of specialization exhibited by the neural circuitry involved in training; a training paradigm that produces alterations in performance on other, unrelated tasks are likely to have altered circuits common to both tasks. Thus, the amount of generalization a perceptual training paradigm elicits provides invaluable information to the researcher regarding the circuits that have been altered by said training, with circuits responsible for processing a range of stimuli exhibiting cross-stimulus generalization and circuits essential for processing a number of related tasks showing cross-task generalization.

It is unclear from the literature whether temporally-based multisensory training paradigms should be expected to show generalization across tasks. Some task generalization has been seen in multisensory short-term passive exposure studies[34,52-55]. Fujisaki and colleagues[52] assessed participants' likelihood of perceiving a range of asynchronous audiovisual pairs as simultaneous and then repeatedly exposed participants to an audiovisual stimulus pair separated by a fixed onset asynchrony for a period of minutes. Re-assessment revealed short-term shifts in participants' perception of simultaneity, and these shifts extended to a pair of audiovisual illusions; notably, these two illusions—the Flash-Beep Illusion[10-11] and the Stream-Bounce Illusion[56]—while unrelated to the exposure task, have a strong basis in multisensory temporal processing, showing a monotonic decline in effect size with deviation from simultaneity. Thus, the authors may be said to have temporarily altered some aspect of multisensory processing underlying all three of the tasks used. In a similar vein, Virsu and colleagues recently reported lasting improvements in accuracy of unisensory and multisensory simultaneity judgments and decreases in mean simultaneity thresholds following practice, but failed to see transfer of training effects across modalities[57]. None of these studies, however, have

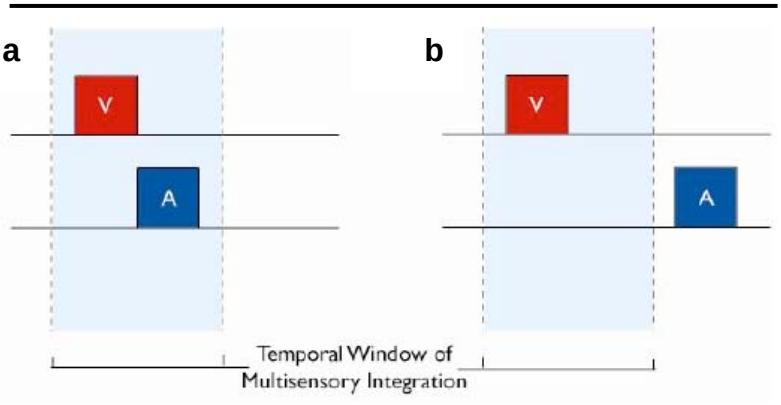

Figure 1 | **The temporal window of multisensory integration**. The dashed lines and light blue shading delimit the temporal window of multisensory integration, in which visual (V) and auditory (A) stimuli are bound into a unified perceptual entity (**a**). When visual and auditory stimuli are sufficiently separated in time, they are processed as independent events (**b**).

attempted to specifically alter the temporal window of multisensory integration by perceptual training.

As described above, the degree to which perceptual training effects generalize across stimuli and across tasks provides important information about the circuits involved in these tasks. In conjunction with these behavioral measures, neuroimaging measures such as fMRI are capable of identifying those brain regions most likely to underlie perceptual phenomena like those described above. As of yet, no neuroimaging data have been produced identifying brain regions altered by perceptual training in a temporally-based multisensory task. It may be hypothesized, however, that the brain regions altered by said training may be the same regions underlying multisensory processing in general and multisensory temporal processing in particular. The literature regarding these brain areas is outlined below.

MULTISENSORY BRAIN NETWORKS

Traditional views of sensory cortical organization posit that sensory information is routed from the thalamus to the primary sensory cortices and then to association cortices where it may be combined with information from other modalities. The focus of much multisensory research has been on these cortical association areas; indeed, the earliest of these have been described as possible loci for the initial binding of multisensory information[58-60]. This early multisensory cortical network appears to be located at the borders between temporal, occipital, and parietal lobes, and includes Brodmann's areas (BA) 39/40 and the posterior superior temporal sulcus (PSTS) as major nodes. These areas have been shown to respond to multisensory stimulation in a variety of different tasks and contexts[26,61-64], which, in conjunction with preliminary data from our lab[30,65] and others[33,66], make them the focus of the current proposal.

The network defined above has been further refined by studies examining the temporal aspects of multisensory processing, which are most germane to the current review. A number of other studies[67-68] have described an expanded network, identifying the multisensory areas above in addition to insula/frontal operculum, dorsolateral medial prefrontal cortex, posteriorparietal cortex, posterior thalamus, superior colliculus, and posterior cerebellar vermisas being involved with multisensory processing in the temporal realm. Because the experiments proposed here will specifically involve measures of multisensory temporal processing, our own analysis will focus on both general multisensory areas and those areas described above that are known to be involved specifically in multisensory temporal function.

Increasing evidence is pointing to early sensory cortices (i.e., unisensory regions) as possible sites for multisensory interactions in addition to these canonically defined multisensory areas[69-77]. While it is unclear whether these interactions are the result of feed-forward, feed-back or lateral connectivity, it seems wise at this juncture to include these areas in any analysis of multisensory processing via neuroimaging.

A thorough description of the plasticity of brain networks involved in multisensory temporal processing is of obvious importance in understanding the characteristics and flexibility of these networks from a basic science perspective. However, as outlined below in the final section of this review, emerging evidence suggests that these questions may also be of utmost importance in establishing the pathophysiology of clinical disorders that have multisensory temporal processing as their basis. Thus, outlining the effects of perceptual training upon these networks brings the hope that training-induced alteration may represent a step toward remediation of these disorders.

CLINICAL IMPLICATIONS

While the study at hand proposes to fill gaps in our knowledge of how multisensory systems react dynamically to changes in the external environment, the conclusions drawn from this research may ultimately extend to the diagnosis and treatment of several disorders. Our lab and others[30,57,78-82] have identified altered multisensory temporal processing in dyslexic readers. Specifically, our lab has described an extended temporal window of multisensory integration in these readers when compared with typical readers. Correspondingly, imaging studies have shown that areas that lie at the borders between occipital, temporal and parietal cortices exhibit significant activation differences in dyslexic readers when compared with typical readers[83-88]. The areas that have been identified in these studies share many similarities with those that make up the early multisensory regions outlined above. Thus, the successful completion of the study proposed here may provide the basis for the investigation of multisensory perceptual training as a viable strategy in the remediation of developmental dyslexia.

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