Dopamine transporter gene susceptibility to methylation is associated with impulsivity in non-human primates

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Abstract

Impulsivity, the predisposition to act without regard for negative consequences, is a characteristic of several psychiatric disorders and is thought to result in part from genetic variation in the untranslated region (UTR) of the dopamine transporter (DAT) gene. As the exact link between genetic mutations and impulsivity has not been established, we used oculomotor behavior to characterize rhesus monkeys as impulsive or calm, and genetic/epigenetic analysis and positron emission tomography to correlate phenotype to DAT genotype, DAT gene methylation, and DAT availability. We found three single nucleotide polymorphisms (SNPs) in the 3’-UTR of the DAT gene, one of which provided a potential site for methylation in the impulsive group. Bisulfite analysis showed that the DNA of the impulsive but not the calm subjects was methylated at one SNP. Because genetic/epigenetic modifications could lead to differences in protein expression, we measured DAT availability using \[^{18}\text{F}]\text{FECNT}\) positron emission tomography and found higher DAT availability in the internal globus pallidus, an output nucleus of the basal ganglia, of the impulsive group. Higher DAT availability lowers DA levels, potentially altering neuronal circuits involved in the initiation of action, thus contributing to the impulsive phenotype. The association between increased methylation in the DAT gene and greater DAT availability suggests that mutations to the regulatory portion of the DAT gene lead to a susceptibility to epigenetic modification resulting in a discrete behavioral phenotype.

Keywords
The propensity to act quickly and without regard for negative consequences is generally referred to as impulsivity. Understanding the neural basis for this type of behavior is of much interest because in many instances such behavior does not seem to be advantageous and, importantly, its incidence in humans correlates with numerous psychiatric disorders (Chamberlain et al. 2005; Moeller et al. 2001).

Impulsivity is thought to result in part from genetic variation in the encoding of the dopamine transporter (DAT; Cook et al. 1995; Gill et al. 1997). The human DAT gene (SLC6A3) contains a variable number of tandem repeats (VNTR) polymorphism in the 3′-untranslated region (UTR), ranging from 3-11 repeats of a 40-base pair sequence (Vandenbergh et al. 1992). The presence of the 10-repeat allele (10R) has been correlated with the occurrence of attention deficit hyperactivity disorder (ADHD; Cook et al. 1995; Gill et al. 1997; Waldman et al. 1998; Daly et al. 1999; Winsberg and Comings 1999), of which impulsivity is a cardinal symptom. In addition, greater DAT availability in the striatum has been associated with ADHD (Dougherty et al. 1999; Dresel et al. 2000; Krause et al. 2000; Larish et al. 2006), impulsivity (Costa et al. 2013), and the 10R (Heinz et al. 2000) and 9R (Jacobsen et al. 2000; Spencer et al. 2013) alleles of the DAT VNTR.

DAT is blocked by methylphenidate, a primary treatment for ADHD that in low doses reduces impulsivity in humans (Greenhill, 2001; Solanto, 1998, 2002) and animals (Rajala et al. 2012). While these correlations suggest that alterations in the DAT gene or its expression mediate impulsive behavior, a direct link has not been established.
For instance, the 10R allele, which is associated with ADHD, is more common in the general population than is the incidence of ADHD (Mitchell et al. 2000).

Rhesus monkeys, the animal model closest to humans available for studies of nervous system function, have an homologous gene encoding DAT with a fixed number of tandem repeats (FNTRs) in the 3’-UTR. A single nucleotide polymorphism (SNP) in the 3’-UTR was found to be suggestive but not predictive of hyperactivity (Miller et al. 2001). This finding, combined with similarities of the Rhesus monkey to the human in structure and function of the frontal part of the brain (Wise, 2008), DA neurotransmission (Berger et al. 1991), and positive response to methylphenidate (Rajala et al. 2012) make it an ideal system for the study of the mechanisms underlying impulsivity. Accordingly, we used oculomotor behavior to characterize rhesus monkeys as impulsive or calm, and genetic/epigenetic analysis and positron emission tomography to correlate phenotype to DAT genotype, DAT gene methylation, and DAT availability.

The results show that the differences in the 3’-UTR of the DAT gene could render some subjects more susceptible to epigenetic modification, which can alter gene expression and, consequently, protein levels. Higher DAT availability lowers DA levels (Heinz et al. 1999), potentially altering the response properties of neuronal circuits involved in the initiation of action, thus contributing to the impulsive phenotype.

Materials and Methods
Subjects. Four 9-12 Kg male, adult (7-14 years) Rhesus monkeys (*Macaca mulatta*) purchased from the Wisconsin National Primate Research Center were studied. These monkeys were not related. The four subjects had participated in earlier studies of the effects methylphenidate on cognitive functions, which took place at least eighteen months before the present study; some of those data have been published (Rajala et al. 2012). Subject selection was based solely on availability and intact eye movement recording implants. The animals were housed individually in two rooms with other monkeys of the laboratory colony in the same hallway of the same facility, permitting rich visual, olfactory, and auditory interactions.

Surgical procedures were carried out under aseptic conditions under general anesthesia. All efforts were made to minimize suffering of the subjects. To ameliorate pain, the monkeys received analgesics for 4-7 days after surgery. The animals were not sacrificed after the experiment. The preparation and surgical details have been published (Populin, 2006). All experimental procedures were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC) and were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

**Experimental setup, eye movement recordings, experimental task, and eye-movement analysis.** The experiments were done in a dimly illuminated sound-proof chamber (Acoustic Systems, Austin, TX). Eye position was measured with the scleral search coil technique (Robinson, 1963; CNC Engineering, Seattle, WA). The signals were digitized (Tucker Davis Technologies, Alachua, FL) and stored for offline analysis.

A memory-guided saccade task with targets at two eccentricities (10° and 20°) and six
delays (1-6 sec in 1 sec steps), presented in random order, was used (Fig. 1). Subjects were presented with a red light emitting diode (LED) straight ahead. A variable time (300-600 ms) after acquiring the fixation LED, a 100 ms target was presented from one of the eight target locations. This was followed by a randomly selected delay period, at the end of which the fixation LED was turned off thereby signaling the subject to make an eye movement to the remembered location of the target. Water was delivered after the completion of successful trials, which required meeting both temporal and spatial criteria. Temporal criteria included maintaining fixation on the LED straight ahead until turned off and executing an eye movement within 700 ms. Spatial criteria involved maintaining eye position with a (4°, 4°) acceptance window set around the initial fixation point for the duration of the delay period and making a saccade with end position falling within a (4°, 4°) acceptance window set around each target. Failure to meet these criteria resulted in termination of the trial at the time the error was made. Thus, trials in which a premature response was detected, in which the subject’s eye position left the acceptance window around the fixation during the delay period for more than 75 ms, were immediately terminated.

They were allowed to perform as many trials as they wanted in each session so they could drink enough water to achieve or exceed the minimum daily intake established by the IACUC. Experimental sessions were terminated after subjects closed their eyes for ten consecutive trials. Supplemental water was provided after sessions in which they did not drink the established minimum. Although this approach resulted in experimental sessions with different numbers of trials (see Table 1), it promoted steady day-to-day performance.

Sample collection, DNA extraction and DAT genotyping. Blood samples were collected
from the four subjects in Monoject vacutainer tubes (Tyco Health Care Group, LP, Mansfield, MA) with 7.5% EDTA (K₃), kept on ice until mixed with RNAlater (Sigma-Aldrich, St. Louis, MO), and then stored at -80°C. The identity of the subjects was unknown to IZ and MLE, who carried out this part of the project. Genomic DNA was isolated using GenElute™ Blood Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO). The quality and quantity of DNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). PCR amplification was conducted in a 25 μL reaction volume consisting of 50 ng of genomic DNA, 100 ng of each primer, 200 μM of each deoxynucleoside triphosphate (1 μL; Promega, Madison, WI), 2.5 μL of 10× PCR buffer, 1.5 units of Taq DNA polymerase (New England Biolabs Inc, Ipswich, MA), and molecular biology grade water (Sigma-Aldrich, St. Louis, MO). Primers used for genotyping were reported previously (Miller et al. 2001): U13: 5' TGTTCAGAGGCATTGGAG 3'; L12: 5' AAAAAGCCATTCGCAAACAT 3'. The purified bands were used for Sanger sequencing according to manufacturer's protocols. Each sample was sequenced twice, first using the forward primer (U13) and the second using the reverse primer (L12). Sequenced samples were run on an ABI 3730xl DNA Analyzer. Sequence data were displayed using FinchTV Version 1.4.0 (Geospiza, Seattle, WA).

Bisulfite genomic modification and sequencing. Methylation was measured using DNA extracted from cells in the blood in order to preserve our subjects for additional studies. Similar methods are used in human studies, which have shown that peripheral DNA methylation correlates with methylation patterns in the brain (Horvath et al. 2012), and with measures of brain protein synthesis (Wang et al. 2012).

To quantify DNA methylation, two micrograms of genomic DNA per subject were bisulfite
converted, which changed unmethylated cytosine into uracil, using the Imprint DNA Modification kit (Sigma-Aldrich, St. Louis, MO). Because DNA methylation can occur on either one or both alleles of the DAT gene, the different alleles were expressed separately in individual bacterial colonies. Modified DNA was purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and eluted in 20 μL H2O. Primers for modified DNA amplification were designed manually and had the following sequences: DAT1_BS_F222: GAGGTATTGGAGGATAGGGGTTT, DAT1_BS_R222: AAAAAATAAATTTCCATTTAAATCTCTTCTTATAAAA. The target region was PCR amplified using 2 μL of bisulfite-modified DNA. Nested PCR was conducted using 2 μL of the first-round-PCR products (DAT1_BS_F223: GTTTAGGGTTATGGTTACGGGTTTT, DAT1_BS_R223: CCAAACATTTATAATCTACAAACTACATAA). Nested PCR products were purified from 1.5% agarose gel using a QIAquick Gel extraction kit (Qiagen, Valencia, CA), and cloned into pGEM®-T Easy Vector Systems (Promega, Madison, WI). Cloning vectors were transformed into JM109 competent cells (Promega, Madison, WI), which were plated onto LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37°C. White colonies were selected and amplified in broth medium. Plasmids, including the pGEM®-T Easy Vector, were isolated using PerfectPrep Spin Mini Kit (5 PRIME, Gaithersburg, MD). Plasmids were amplified using the following primers in a 25 μL reaction: pUC/M13-For: AACGCCAGGGTTTTCCCAGTCAC; pUC/M13-Rev: TCACACAGAAACAGCTATGAC. Eighty nanograms of PCR product were used for Sanger sequencing as described earlier, using the T-7 primer (TGTAATACGACTCACTATAGGG). The methylation status was determined from the DNA sequence of several individual colonies for each subject and the percentage of methylated
colonies was calculated relative to the total sequenced colonies for each subject.

**Positron Emission Tomography (PET) scans.** After the behavioral and genetic parts of the study were complete, DAT availability was measured in all four subjects using the cocaine analog $[^{18}\text{F}]2\beta$-carbomethoxy-3$\beta$-(4-chlorophenyl)-8-(2-fluoroethyl)-nortropane ($[^{18}\text{F}]\text{FECNT}$; Murali et al. 2013), a radioligand selective for DAT (Goodman et al. 2000). The scanning protocol has been published (Converse et al. 2013). Briefly, subjects were sedated with a light dose of ketamine (5 mg/kg IM) approximately 25 min before injection of the radiotracer and transported from the vivarium to the imaging facility in a climate-controlled vehicle. The trip took approximately 5 min. Upon arrival the animals were intubated and gas anesthesia was started (1-2% Isoflurane). A catheter was inserted into the cephalic vein for administration of the $[^{18}\text{F}]\text{FECNT}$ and fluids during recovery from anesthesia. Subsequently, the monkeys were positioned in a microPET P4 scanner (Siemens, Knoxville, TN), and a $^{57}\text{Co}$ transmission scan was acquired, after which a 150 minute emission scan began. $[^{18}\text{F}]\text{FECNT}$ was injected IV 60 sec after the start of the scan. The injected radioactivity was $191\pm13$ MBq (mean, SD, n = 4).

Upon recovery from anesthesia the monkeys were returned to their quarters using the same transport method. Scans were completed over two days with scanning order counter-balanced across the two groups (2 scans per day; 1 impulsive and 1 calm subject per day in opposite order). The behavioral and genetic classification of the subjects was unknown to AKC who performed the PET scans and the image analysis.

**Image data processing, pharmacokinetic modeling, and statistical analysis.** Images were aligned to an $[^{18}\text{F}]\text{FECNT}$ template (Converse et. al, 2013) and time-activity curves were determined for anatomically defined regions of interest: internal division of the globus pallidus
(GPI), external division of the globus pallidus (GPe), and areas of the frontal cortex; and regions
defined by radioactivity in the template image: the substantia nigra (SN), ventral tegmental
area (VTA), and the striatum (Str; Converse et al. 2013). To better locate the SN and VTA, the
anatomically defined regions were warped to fit the region in the [$^{18}$F]FECNT template image
defined by the contour at half of the peak radioactivity concentration found in the midbrain.
The [$^{18}$F]FECNT template was the average FECNT radioactivity image of the control subjects
from Converse et al. 2013. [$^{18}$F]FECNT binding was determined using the Logan graphical
reference tissue method with a cerebellar reference region.

Differences in regional DAT availability between the calm and impulsive groups were
examined with analysis of covariance (ANCOVA) where the between subjects' variable was
Group (Calm/Impulsive) and Age was the covariate; the subjects’ phenotype was known to
JBH, who performed the statistical analysis.

RESULTS

Behavioral phenotype. While training monkeys to perform a memory-guided saccade task
configured to create a high level of uncertainty, large differences in performance among the
subjects were observed (Fig. 1). Specifically, two subjects frequently responded prematurely
before the offset of the fixation LED in many trials. The other two subjects were better able to
wait for the signal to respond. The inability of the former subjects to wait for the appropriate
time to respond, which led to immediate termination of the trials and loss of reward,
resembled the type of responses that characterize individuals clinically labeled as impulsive,
i.e., the tendency to act without regard for negative consequences (Moeller et al. 2001).
Therefore, we operationally refer to these subjects as “impulsive”, and we refer to the others, who were able to better follow the rules of the task, as “calm”.

The responses of one of the impulsive subjects, MI, are illustrated in Figure 2A, correct (blue) and incorrect (red). They were characterized by reflexive reactions shortly after the presentation of the target (arrow), followed by numerous small eye movements during the delay period, during which steady fixation was required. The responses of one of the calm subjects, SW, in contrast, illustrate that he was able to wait for the offset of the fixation LED in most trials, and made fewer premature responses (Fig. 2B).

The percentage of premature responses made by each subject during testing sessions confirmed that they could be classified into two groups, calm and impulsive (Fig. 2C). The impulsive group made a significantly greater percentage of premature responses compared to the calm group ($F_{1,11.16} = 33.428, P < 0.001$, partial $\eta^2 = 0.71$, Linear Mixed Model; Fig. 2C), while the behavior of the subjects within each group was indistinguishable (calm: $F_{1,8} = 1.370, P = 0.275$; impulsive: $F_{1,7} = 1.315, P = 0.289$, Linear Mixed Model).

Both groups made the majority of their premature responses shortly after the presentation of the target (time = 0 ms), but the distribution of the timing of these responses was different between the two groups (Fig. 3A,B). The inset in Figure 3B compares Gaussian fits to the normalized data using the following model: $f(x) = a_1 \exp(-(x-b_1)/c_1^2) + a_2 \exp(-(x-b_2)/c_2^2)$; the data were normalized to compare the shape of the distributions. The $c_2$ coefficient was significantly larger in the fit of the impulsive 1,918 [CI: 1610, 2,225]) compared to the calm 732.4 [CI: 621.5, 841.2], indicating that this group made more premature responses during the delay period. Interestingly, there was no significant differences in the latency of
correct responses between the two groups (P > 0.05).

In addition, premature responses were evenly distributed throughout the length of the experimental sessions, as shown by their cumulative functions (Fig. 3B). The $R^2$ coefficients (Subject MI: $0.9966 \pm 0.0030$; SH: $0.9918 \pm 0.0079$; GO: $0.9753 \pm 0.0258$; SW: $0.9777 \pm 0.0274$) indicate that the functions are well-described by straight lines, demonstrating that the subjects did not make more premature responses early in the experimental sessions because they were more thirsty, and thus less able to withhold their responses, or later in the sessions because they were more tired, thus more prone to making errors.

It could be argued that these behavioral differences resulted from variations in the length of training provided to these subjects, but this was not the case (Table 1). Subjects SH and SW, who were classified as impulsive and calm, respectively, required nearly the same number of practice trials to reach a preset level of performance, defined as the point at which they earned their daily allowance of water, whereas subject MI, who was classified as impulsive, required nearly twice as many practice trials. Subject GO, who was classified as calm, on the other hand, required the fewest number of practice trials.

**Genotyping and epigenetics.** It was hypothesized that the difference between the phenotypes might be explained instead by variation in the DAT gene. Blood samples were obtained from all subjects, then the FNTR of the 3’-UTR region of the DAT gene was amplified and sequenced under double blind conditions (Fig. 4A). Three SNPs were found: the first and the second were either adenine (A) or guanine (G), while the third was either thymidine (T) or guanine (G) and was identical to the SNP previously reported in monkeys (Miller et al. 2001; Fig. 4B). Each SNP was examined in each subject for differences in the proportion of
PCR products with each version of the two possible alleles, the distribution of which, at both the first and third SNP sites, was significantly different between the calm and impulsive groups (P < 0.0001 at SNP1 and P < 0.010 at SNP3, Fisher’s two-tailed exact test; odds ratio (OR) = 0.05, 95% confidence interval (CI) 0.00 - 0.35 and OR = 0.24, 95% CI 0.00 - 0.89, respectively; Table 2). Cramer’s Φ was 0.69 and 0.50, respectively, indicating a large effect of both SNP sites, despite the small sample size. Specifically, none of the samples from the calm subjects had a G at SNP1 and none of the samples from the impulsive subjects had a T at SNP3 (Table 2). There was no significant difference between the groups at SNP2 (P = 0.497, Fisher’s exact test, two-tailed, OR = 0.24, 95% CI 0.01 - 5.54; Φ = 0.22).

Interestingly, all three SNPs were immediately downstream of a cytosine (C), resulting in either a CpA, CpT, or CpG dinucleotide, the last of which is a substrate for DNA methylation, an epigenetic modification that can alter gene expression (Moore et al. 2013, Robertson, 2005). In addition, 14 other CpG dinucleotides were found in the FNTR region (Fig. 4C). The methylation status of the identified CpG sites was examined in the DNA extracted from blood cells. Each SNP was examined in each subject for the proportion of alleles that were methylated. The methylation of the CpG at SNP1 (site 4) was significantly different between the two behavioral phenotypes. While the methylation at SNP1 was different between the two subjects in the impulsive group (P < 0.05, Fisher’s exact test, two-tailed), it was completely absent in both subjects of the calm group as a result of the lack of a CpG (P = 0.022, Fisher’s exact test, two-tailed; OR = 32.07, 95% CI 1.60 - 643.44; Φ = 0.57, Fig. 4D). There were no significant differences in methylation at the 14 non-SNP CpG sites between the groups.

**PET imaging of DAT.** The methylation status in the regulatory region of a gene may affect
expression by altering the binding of transcription factors (Moore et al. 2013; Robertson, 2005; Shumay et al. 2010). The differences in methylation between the impulsive and calm groups could result in differences in the expression and thus the availability of DAT, resulting in specific behavioral phenotypes. Accordingly, DAT availability was measured using PET after injecting $[^{18}F]$FECNT, a selective radioligand for DAT (Goodman et al. 2000).

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The mean radioactivity from all four subjects at two different coronal slices illustrates the qualitative distribution of DAT binding and the defined regions of interest (Fig. 5). Because DAT availability decreases with age in humans (Bannon et al. 1992; Costa et al. 2013; Dougherty et al. 1999; Volkow et al. 1994, 1996), an analysis of covariance (ANCOVA) was performed with age as a covariate. The impulsive group had a significantly higher level of DAT available in the GPi ($F_{1,1} = 5791.60, P < 0.01$, partial $\eta^2 = 1.00$), the output nucleus from the basal ganglia to the thalamus (Table 3). A similar trend across groups was found for the GPe ($F_{1,1} = 87.25, P = 0.068$, partial $\eta^2 = 0.99$). As in humans (Ciliax et al. 1999), the highest DAT availability was recorded in the striatum (Fig. 5). However, unlike in humans (Winsberg and Comings, 1999; Dougherty et al. 1999; Dresel et al. 2000; Krause et al. 2000), there was no significant difference between the groups in this region (Table 3).

**DISCUSSION**

The data show in the same subjects a linkage between the occurrence of an impulsive phenotype, increased methylation of SNP1 (site 4), and increased DAT availability in the GPi. These findings indicate that mutations in the regulatory region of the DAT gene are substrates for potential epigenetic modification, as suggested by modeling work in the human SLC6A3
The result of these alterations could increase DAT protein levels, which in turn would alter the normal function of basal ganglia/prefrontal cortex circuits leading to impulsive behavior, a hallmark of ADHD and other psychiatric disorders.

The modified memory guided-saccade paradigm used to characterize the subjects' behavioral phenotypes is a very sensitive assay for the characterization of impulsive behavior. The characteristics of eye movements (e.g., latency and kinematics) have been shown to correlate with impulsive behavior in humans (Cirili et al. 2011). Other measures, such as direct observations of the subjects in their home cages (e.g., Miller et al. 2001), are not likely to detect subtle but consistent actions indicative of impulsivity, such as the small eye movements documented in this study (Fig. 2A), and may introduce potential confounding effects. The intruder paradigm, for instance, reveals that the presence of a human in the same room with a non-human primate evokes distinct emotional and behavioral responses (Kalin et al. 1991).

It is important to emphasize that the phenotypic classification obtained with the memory-guided saccade task was not dependent on the extent of the behavioral training. The tendency of the impulsive subjects to respond prematurely even after additional training was consistently observed throughout the course of all the experimental sessions, ruling out, therefore, concerns about motivation and fatigue as drivers of premature responses (Fig. 3).

This type of paradigm, allows for not only the detection of very subtle behavioral manifestations of impulsive behavior (Fig. 2) but also the collection of large datasets comprising literally tens of thousands of trials (Table 1). However, it requires long periods of training, which preclude testing large numbers of subjects. The sample size of this study is not an exception but the norm in studies of awake behaving monkeys involving combinations of
cognitive and molecular measures (e.g., Liu et al. 2004). Nevertheless, to allay any concerns, measures of effect size appropriate for each statistical test, which summarize the results with respect to the population (Cohen, 1992), were computed and revealed large effects for all statistically significant results. Therefore, the large effect sizes reported here render moot any concerns about under-powered statistical tests and warrant drawing conclusions from the present data.

Chronic treatment with methylphenidate is known to upregulate the expression of DAT (Shumay et al. 2010). Although the monkeys in this study had participated in previous studies of the effects of methylphenidate on cognitive function (Rajala et al. 2012, and unpublished observations), they were not treated chronically and were only exposed to the drug in single weekly treatments that ended more than eighteen months before this study. Such exposure, therefore, is unlikely to have had a long-lasting effect that could have affected the present results.

The higher availability of DAT documented in the GPi of the impulsive monkeys is consistent with the higher levels of DAT found in the striatum of humans diagnosed with ADHD (Dougherty et al. 1999; Dresel et al. 2000; Krause et al. 2000; Larish et al. 2006) and impulsivity (Costa et al. 2013). It will be important to determine in future studies if differences in DAT availability between phenotypes are limited to nuclei that comprise the basal ganglia (e.g., Dougherty et al. 1999 and the present study), or if they extend to other brain structures where DAT is expressed, such as the prefrontal cortex.

Polymorphisms within the 3′-UTR of both the human and monkey DAT genes have been shown to alter DAT expression, but no specific mechanism has been identified (Miller and
Because DNA methylation is thought to silence gene expression by preventing transcription factors from binding to DNA (Robertson, 2005), it is hypothesized that the greater availability of DAT in the impulsive group results from methylation preventing the binding of a transcription factor produced by a suppressor gene, which reduces DAT expression. An example of this is Hesr1/Hey1, which binds to the human DAT gene (SLC6A3) and down-regulates its expression in human neural cell lines (Fuke et al. 2005). Variability in the encoding of such transcription factors could also contribute to an impulsive phenotype (Fuke et al. 2005).

The occurrence of ADHD and increased DAT availability have been associated with the 10R allele of the VNTR polymorphism in the 3′-UTR of the human DAT gene (SLC6A3; Cook et al. 1995; Daly et al. 1999; Gill et al. 1997; Heinz et al. 2000; Waldman et al. 1999; Winsberg and Comings, 1999). However, this allele is the most prevalent in the general population, in which the occurrence of ADHD is much lower (Mitchell et al. 2000). The present results from the monkey suggest that a polymorphism may provide a substrate for epigenetic changes, which could contribute and/or potentially account for this discrepancy.

The environment may influence genetics via epigenetic mechanisms such as DNA methylation, both during development (van Heesbeen et al. 2013) and dynamically throughout life (Moore et al. 2013). It is unlikely, however, that environmental conditions throughout the duration of the study confounded the results because all the monkeys were housed under virtually identical conditions.

While differences in DAT encoding and availability are not solely responsible for impulsive behavior, the question remains as to how might higher levels of DAT in the GPi alter neural
processing and thereby contribute to an impulsive phenotype. Dopamine signals, which encode reward prediction error (Schultz et al. 1997) and are modulated by uncertainty (de Lafuente and Romo, 2011), can facilitate behavioral responses to relevant external and internal stimuli. Increased levels of extracellular DA in the striatum increase evoked responses relative to spontaneous activity and, therefore, increase signal-to-noise ratio (Kiyatkin and Rebec, 1996; Nicola et al. 2000; Rolls et al. 1984). Accordingly, higher availability of DAT should result in lower levels of extracellular DA (Heinz et al. 1999) and decreased signal-to-noise ratio, which, in turn, should lead the circuit to reach the threshold for responding more easily. The increased levels of DAT in the Gpi documented here could facilitate the initiation of actions in response to external (or internal) signals via the direct (GO) pathway, and faster termination of ongoing actions, facilitating the initiation of subsequent actions, via the indirect (NO-GO) pathway. Essentially, we hypothesized that lower levels of DA brought about by higher DAT availability should result in reaching the threshold for responding more easily and contributing to impulsive behavior. The fact that the experimental task used to characterize the impulsive and calm phenotypes in this study was purely oculomotor (Fig. 1) seems to contradict the accepted view of the motor systems’ specific output pathways of the basal ganglia. Specifically, that the initiation of saccadic eye movements relies on output from the substantia nigra pars reticulata (SNr) not the Gpi (Hikosaka and Wurtz, 1983a,b; 1985; Hikosaka et al. 2000). However, recent single unit recordings from neurons in the Gpi and GPe of monkeys reveal activity related to different aspects oculomotor behavior (Hong and Hikosaka, 2008), supporting the hypothesis that this output of the basal ganglia is also involved in the generation and control of eye movements (Kato and Hikosaka, 1995; Shin and Sommer, 2010;
In conclusion, our data suggest that epigenetic regulation of DAT gene expression may provide a major unexplored component to the manifestation of specific behavioral phenotypes known to be associated with psychopathologies (Moeller et al. 2001), and offer a new perspective for investigating the basic mechanisms underlying dopaminergic neurotransmission.

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Disclosures

The authors declare no conflicts of interest, financial or otherwise.
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Figure 1. Experimental task. The subject was first required to fixate on a light emitting diode (LED) straight ahead (1). During fixation, one of eight targets was presented for 100 ms elsewhere within the oculomotor range (2). Subsequently, a delay period of randomly determined duration (1-6 sec) was imposed (3). The subject had to maintain fixation for the entire duration of this period. Lastly, upon offset of the fixation LED, which constituted the signal to respond, the subject was expected to make a saccadic eye movement to the remembered location of the target (4).

Figure 2. (A) Representative examples of saccadic eye movements from impulsive subject MI. The vertical or horizontal component of eye movements representing the main direction of movement is plotted as a function of time and synchronized to target presentation (0 ms). Blue traces represent trials in which the subject responded at the appropriate time and red traces represent trials in which the subject responded prematurely. The signal to respond for all six delays is illustrated above the abscissa. In this example the target was located at (0°, 20°) and premature responses comprised ~50% of all trials. Note the small eye movements recorded shortly after the presentation of the target (black arrow). This reflexive type of movement, which was present in most trials involving targets in all directions and amplitudes, best illustrates the impulsive nature of this subject’s responses. (B) Representative example of performance by calm subject SW. Note that most eye movements were performed in a timely manner, presenting a stereotyped pattern defined by the six different delays. (C) Percent of premature responses measured across 4-6 experimental sessions for each subject. The impulsive group made a significantly larger percentage of premature responses (F_{1,11.16} =
33.428, P < 0.001, Linear Mixed Model), but the subjects within each group were not significantly different (calm: F_{1,8} = 1.370, P = 0.275; impulsive: F_{1,7} = 1.315, P = 0.289, Linear Mixed Model).

**Figure 3.** (A, B) Distribution of response time for both correct and premature responses for the calm and impulsive groups, respectively. The inset in (B) compares the Gaussian fits to the normalized data. The resulting coefficients show that the bell of the Gaussian distribution fit to the data of the impulsive group was significantly wider than that of the calm group (arrow), indicating that the impulsive group made more premature responses during the delay period. Both groups made most premature responses shortly after the presentation of the target, but the proportion of premature responses to correctly-timed trials was greater in the impulsive group. (C) Cumulative functions of the number of premature responses made during the course of testing sessions. Linearity was assessed by computing the average R^2 ± 95% confidence interval.

**Figure 4.** (A) Schematic representation of the DAT gene of the Rhesus monkey. (B) Three single nucleotide polymorphisms (SNPs) found in the 12-repeat fixed number tandem repeats (FNTR) sequence, revealed by the double peaks at SNP1 - G/A, SNP2 - G/A, and SNP3 - G/T. (C) Nucleotide sequence of the FNTR region. Seventeen CpG dinucleotides were found with the three SNPs occurring at CpG numbers 4, 6, and 17 (magenta). (D) Methylation status of SNP-containing CpGs expressed as percent of all methylated clones for each subject in each of the two groups. The SNP1 - CpG_4 site was significantly more methylated in the impulsive...
group compared to the calm ($P = 0.022$, Fisher's two-tailed exact test).

Figure 5. [$^{18}$F]FECNT PET images. Coronal slices of mean radioactivity ($n=4$) computed across the entire 150 minute scan. Contours delineate regions of interest defined by radioactivity for striatum (Str), by anatomy for the globus pallidus internal (GPI), and by a combination for the substantia nigra (SN) and ventral tegmental area (VTA). Sections taken at 3.0 (a) and 11.5 (b) mm posterior to the anterior commissure of the corpus callosum (AC).

Table 1. Behavioral classification and number of practice and testing trials performed by each subject.
<table>
<thead>
<tr>
<th>Group</th>
<th>Subject</th>
<th>Number of practice trials</th>
<th>Number testing trials</th>
<th>Number of testing sessions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calm</td>
<td>SW</td>
<td>38,980</td>
<td>4094</td>
<td>6</td>
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<tr>
<td></td>
<td>GO</td>
<td>12,639</td>
<td>2051</td>
<td>4</td>
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<tr>
<td>Impulsive</td>
<td>SH</td>
<td>38,734</td>
<td>1102</td>
<td>4</td>
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<tr>
<td></td>
<td>MI</td>
<td>67,855</td>
<td>2726</td>
<td>5</td>
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</table>

Table 2. Genotypes of the four subjects at the three SNPs. The haplotypes are listed for each animal at each single nucleotide polymorphism (SNP) in the DAT fixed number of tandem repeat region (FNTR). The calm and impulsive groups differed significantly in
genotype at SNP1 and SNP3 (*P < 0.01, **P < 0.001, Fisher’s exact test). The subjects
within each group were not significantly different from each other.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Phenotype</th>
<th>SNP1**</th>
<th>SNP2</th>
<th>SNP3*</th>
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</thead>
<tbody>
<tr>
<td>SW</td>
<td>Calm</td>
<td>A/A</td>
<td>A/G</td>
<td>T/G</td>
</tr>
<tr>
<td>GO</td>
<td>Calm</td>
<td>A/A</td>
<td>G/G</td>
<td>T/G</td>
</tr>
<tr>
<td>SH</td>
<td>Impulsive</td>
<td>G/G</td>
<td>G/G</td>
<td>G/G</td>
</tr>
<tr>
<td>MI</td>
<td>Impulsive</td>
<td>A/G</td>
<td>G/G</td>
<td>G/G</td>
</tr>
</tbody>
</table>

Table 3. Binding potential of FECNT to DAT measured from PET and results of
statistical analysis. ROI = region of interest; BP (mean) = adjusted binding potential
mean; GPi = internal globus pallidus; GPe = external globus pallidus; SN = substantia
nigra; VTA = ventral tegmental area. The adjusted means represent bilateral binding potentials in each region controlling for age.

<table>
<thead>
<tr>
<th>ROI</th>
<th>Impulsive (n=2)</th>
<th>Calm (n=2)</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>BP (mean)</td>
<td>std error</td>
<td>BP (mean)</td>
<td>std error</td>
</tr>
<tr>
<td>GPi</td>
<td>1.659</td>
<td>0.001</td>
<td>1.584</td>
<td>0.001</td>
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<tr>
<td>GPe</td>
<td>6.174</td>
<td>0.222</td>
<td>3.061</td>
<td>0.222</td>
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<tr>
<td>Striatum</td>
<td>8.254</td>
<td>0.169</td>
<td>7.212</td>
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<tr>
<td>SN</td>
<td>2.060</td>
<td>0.142</td>
<td>2.374</td>
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<tr>
<td>VTA</td>
<td>2.283</td>
<td>0.012</td>
<td>2.384</td>
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</table>