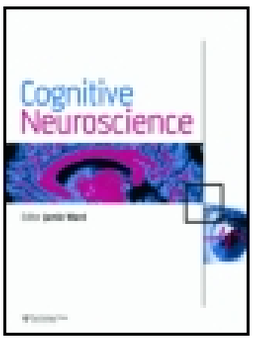

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# Methylation polymorphism influences practice effects in children during attention tasks

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**ABSTRACT**

Epigenetic mechanisms mediate the influence of experience on gene expression. Methylation is a principal method for inducing epigenetic effects on DNA. In this paper, we examine alleles of the methylenetetrahydrofolate reductase (MTHFR) gene that vary enzyme activity, altering the availability of the methyl donor and thus changing the efficiency of methylation. We hypothesized that alleles of the MTHFR gene would influence behavior in an attention-related task in conjunction with genes known to influence attention. We found that seven-year-old children homozygous for the C allele of MTHFR in interaction with the catechol *O*-methyltransferase (COMT) gene showed greater improvement in overall reaction time (RT) and in conflict resolution with practice on the Attention Network Test (ANT). This finding indicates that methylation may operate on or through genes that influence executive network operation. However, MTHFR T allele carriers showed faster overall RT and conflict resolution. Some children showed an initial improvement in ANT RT followed by a decline in performance, and we found that alleles of the dopamine beta-hydroxylase (DBH) gene were related to this performance decline. These results suggest a genetic dissociation between improvement while learning a skill and reduction in performance with continued practice.

**KEYWORDS**

Attention network test; Conflict; MTHFR; DBH; COMT; Epigenetic



Most skills improve in speed and accuracy with practice (Fitts & Posner, 1967). What role does gene expression play in these changes? According to a recent paper: "Emerging evidence suggests that epigenetic mechanisms including DNA methylation are essential regulators of synaptic plasticity and experience dependent behavioral change" (Day et al., 2013).

Current research has shown a link between individual differences in experience and gene expression. For example, a burgeoning literature links the expression of the glucocorticoid receptor gene and early life stress (McGowan et al., 2009; Turecki & Meaney, 2014). Investigation into the mechanism revealed epigenetic processes, including direct modification of genes and structural genomic proteins. Similarly, research in the field of learning and memory has identified a critical role of epigenetics in the regulation of learning-related genes and performance (for a review, see Guan, Xie, & Ding, 2015). There are several forms of epigenetic change, and among the most studied involve changes related to histones in memory formation (Pavlopoulos et al., 2013; Peleg et al., 2010) and to DNA methylation (Day et al., 2013; McGowan et al., 2009). Our paper examines the possible role of methylation in human skill learning.

One gene that may influence the efficiency of methylation is the methylenetetrahydrofolate reductase (MTHFR) gene, which has been related in human studies to overall levels of genome methylation (Friso et al., 2002; Llanos et al., 2015; Stern, Mason, Selhub, & Choi, 2000). Mice deficient in MTHFR expression show evidence of reduced methylation (Chen et al., 2001). Individuals homozygous for the T variant (677 C > T) of MTHFR have a significantly reduced level of enzymatic activity translating to lower general methylation levels in the genome of peripheral leukocytes and lower red blood cell folate levels (Stern et al., 2000). Studies of adult schizophrenic patients and healthy individuals have shown that the presence of this polymorphism blunts the activity of the prefrontal cortex, reduces the response to errors, and reduces activity in the dorsal anterior cingulate (Roffman, Gollub, et al., 2008; Roffman, Nitenson, et al., 2011; Roffman, Weiss, et al., 2008). A population of childhood leukemia

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survivors showed differences in attention and processing speed in relation to MTHFR genotype (Kamdar et al., 2011). Since the 677T allele appears to limit both the rate of enzyme activity and genomic methylation, it may also limit the rate at which learning-related genes can be regulated epigenetically. Our view of the specific process by which MTHFR influences performance is described below[1].

It has recently been shown in rats that the rate of learning a rewarded task is related to the efficiency of methylation in the ventral tegmental area (Day et al., 2013). Based on these findings, we hypothesized that improvements in overall reaction time (RT) and in the time to resolve conflict on a task would depend in part upon efficient methylation in the dorsal anterior cingulate. Practicing the Attention Network Test (ANT; Fan, McCandliss, Sommer, Raz, & Posner, 2002) has been shown to improve reaction time with trials (Ishigami & Klein, 2010) and to activate the dorsal anterior cingulate (Fan, McCandliss, Fossella, Flombaum, & Posner, 2005). The ANT requires participants to press a key in the direction indicated by a target that is presented with surrounding flankers (Eriksen & Eriksen, 1974) that can be congruent with (point in the same direction), or incongruent with (point in the opposite direction) the target (see Figure 1). The difference between incongruent and congruent trials has been shown to correlate with activation of the dorsal anterior cingulate and is thought to be a measure of the ability to resolve conflict (Fan, McCandliss, et al., 2005).

Faster reaction times, such as those occurring with repeated performance of a task, have long been thought to involve selection of the most appropriate action to improve the speed of response (Fitts & Posner, 1967). Thus, we expected to find an improvement in reaction time in our children. We hypothesized that if this improvement involved methylation, it would be lower for those children who had the T allele of MTHFR in comparison to those with the CC genotype, because the T allele would be associated with less-efficient gene regulation during the learning process. Thus, we hypothesized that those children possessing the T variant of MTHFR would show less improvement over trials in the ANT than those homozygous for the C allele. By examining the difference in RT between incongruent and congruent trials, we could also examine the influence of variants of MTHFR on the resolution of conflict. MTHFR has previously been shown to interact with catechol *O*-methyltransferase (COMT), an enzyme associated with executive function, on the performance of executive tasks (Kontis et al., 2013; Roffman, Gollub, et al., 2008, Roffman, Weiss, et al., 2008). If the conflict effect of the ANT was related to genetic differences, we could examine their influence on another executive attention task to see if the same held true for that task. Finally, we chose to explore genetic variation in the gene encoding dopamine beta-hydroxylase (DBH), related to sustained attention, to see if it might be related to the small upswing we observed in RT between Days 2 and 3.

In short, we hypothesized that the alleles of the MTHFR gene would interact with COMT to influence learning of the skill in the form of faster RT and better resolution of conflict over sessions. In addition, we predicted that a tendency to slow down in the later stages of learning would be influenced by alleles of the DBH gene related to reduced attention.

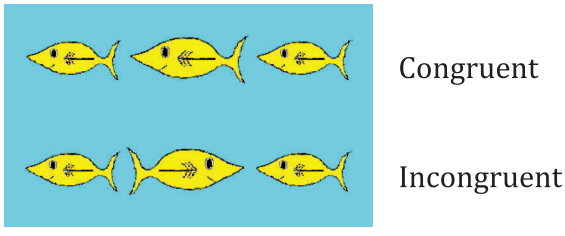


**Figure 1.** In the child ANT, the target is an animal (fish, bird, or mouse) in the center of the display that points left or right. The surrounding flankers either point in the same or opposite direction. The time to resolve conflict is a subtraction of congruent RTs from the incongruent RTs. Figure 1 shows the fish display.

## Materials and methods

Subjects: 70 children, 34 from our ongoing longitudinal study (Rothbart, Sheese, Rueda, & Posner, 2011), were recruited at 7–8 years of age ($M$ = 93.4 months, $SD$ = 13.1 months, 63% male). Three children were re-recruited after an absence in participation in the study, and the remaining 31 had attended the previous year's session. Thirty-six were newly recruited to the study. Of the children, 75.7% were white, 10%

[1]A key enzyme in the production of the methyl donor for methylation reactions is MTHFR (methylenetetrahydrofolate reductase) which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which subsequently donates a methyl group to homocysteine. This methyl group is ultimately used in cellular methylation reactions, including epigenetic modification of other genes.

Hispanic, 2.9% African American, 1.4% Asian, 1.4% with Native American heritage, and the remaining 8.6% were of mixed ethnicity. Genetic information was collected from 68 subjects. Written consent was obtained in the first session for participation in the study, and procedures were approved by the Institutional Review Board of the University of Oregon.

## Behavioral measures

### ANT training

Three training sessions were attended within a two-week period, separated by at least one day. Each session lasted between one-half and one hour. Sixty-eight of the participants attended all three sessions, two attended only one session. Two-thirds of the data were missing for one child's session, and the remaining one third was used to represent this time point. The child version of the ANT was administered as a computer game. Each session began with a brief practice before the testing process. Three sets of 32 targets were displayed as different animals, half pointing left and half right, with similar flankers either congruent or incongruent with the target. Prior to the target, one of four cue conditions were presented in randomized order: center cue, double cue, spatial cue at the target location, and no cue. The flankers were incongruent to the target for half of the trials and congruent for the other half. The child pressed one of two buttons to designate the direction in which the target animal's head pointed, and RT and errors were recorded. Median reaction times for correct trials longer than 100 milliseconds were computed for each child and the overall means of these medians are presented in the Tables and Figures.

### HTKS task

The head-toes-knees-shoulders (HTKS) task (McClelland et al., 2014; Ponitz et al., 2008) is a modification of a "Simon Says" game, where points are earned for correct movement toward a body part. This is a conflict task, since the child is instructed to touch a specific body part different than the one named (e.g., in response to the command "touch your knees," the child touches their shoulders during a correct trial). The HTKS task challenges attention, inhibitory control, and working memory. The HTKS version used in this study had three blocks of 10 trials, where the first block involved two body parts and two rules relating body part to correct response (e.g., touch shoulders to instruction knee and touch knees to instruction shoulder), the second block added two more body parts (total = 4) and used four rules, and the final block had four body parts and four rules, but the previous instructions were switched to a new arrangement. Each block places increasing cognitive demand on the child. The task was presented over two sessions, where the first block was performed in Session 2 and the remaining blocks in Session 3. In a trial, two points were earned if the child touched the correct body part directly or after pausing. One point was earned if a child touched the correct body part but moved their hands first toward a different body part. The HTKS score is the total number of points out of a possible total of 20 points per block.

### Genotyping

Saliva was collected from 67 participants using Oragene DNA collection kits (DNA Genotek Inc, Ottawa, Canada) and one buccal sample was taken using a swab (total 68). Two subjects did not contribute to the genetic analysis. The samples were processed following the Oragene protocol.

The MTHFR locus was amplified using 10 μM each of the following primers, 5′-CGAAGCAGGGAGCTTTGAGG and 5′-AGGACGGTGCGGTGAGAGTG, and the following conditions: 2 mM each deoxynucleotide, 1.5 mM $MgCl_2$, 1.25 units Taq DNA polymerase (recombinant, Thermo Scientific, USA) with its 1x $(NH_4)SO_4$ buffer, and approximately 10 ng of DNA. The amplification conditions were as follows: 94°C 3 min, 40 × (94°C 30 sec, 56° C 30 sec, 72°C 30 sec), 72°C 3 min. The resultant products were digested with HinfI (NEB, USA) at 37°C and size-separated on a 1.5% agarose gel to reveal 233 bp (C allele) and 57/176 bp (T allele) products.

DBH was amplified with 10 μM each primer (Cubells et al., 1998), with the following differences from the MTHFR amplification: 3 mM $MgCl_2$ and a 60°C annealing temperature. The products were digested with EcoNI (NEB, USA) and gel-separated to identify 207 bp (A allele) and 38/169 bp (G allele) fragments. The COMT genotype and haplotype were determined following Voelker et al. (Voelker, Sheese, Rothbart, & Posner, 2009).

In our sample of 68 genotyped subjects, the MTHFR polymorphism (rs1801133) had a minor allele frequency of 33.1% (T). The DBH variation (rs1108580) had a minor allele frequency of 45.6% (A), and the COMT variation (rs4680) had a frequency of 44.1% (G). These proportions did differ from global

frequencies, but were not significantly different than those found in North America (HAPMAP-CEU, a cohort from Utah with European heritage).

## Results

### Behavioral effects

Adult studies have shown clear improvement in reduced overall reaction time over several sessions in both ANT reaction times and measures of conflict, obtained by subtracting congruent RT from incongruent RT (Ishigami & Klein, 2010). Table 1 shows that in our study average overall reaction times declined from Day 1 to Day 2 and slightly increased from Days 2 to 3. An Analysis of Variance showed a difference in reaction time between sessions ($F(2, 203) = 4.65$, $MSE = 64435.05$, $p = .011$) and significant change in reaction time over sessions within subjects ($F(2, 134) = 16.08$, $MSE = 65332.61$, $p < .001$). A significant change in reaction time was found between Sessions 1 and 2 ($F(1, 67) = 47.15$, $MSE = 232420.59$, $p < .001$). There was no significant difference between Sessions 2 and 3 ($F(1, 67) = 1.23$, $MSE = 8798.56$).

The mean conflict score for each session of the ANT is also shown in Table 1. Lower scores represent better performance in resolving conflict. In a repeated measures analysis, conflict scores improved significantly within subjects from training ($F(2, 134) = 3.21$, $MSE = 5498.54$, $p = .043$). The contrast between Sessions 1 and 2 showed a significant improvement ($F(1, 67) = 5.30$, $MSE = 17713.33$, $p = .024$), while between Sessions 2 and 3 there was little change ($F(1, 67) = .036$, $MSE = 97.68$).

In previous studies with young children, we found that repeating a task over many trials often led first to a decrease in RT due to practice, but later an increase as the children began to find the task boring and tiresome (Kieras, 2006). We did not assume that positive practice effects ended, but that they were not sufficient to overcome slowing due to reduced motivation. In support of this general idea, we found no significant correlation between RT change from Days 1 to 2 and change from Days 2 to 3 ($r(68) = .019$). In the present study, we could view the scores over the three days as reflecting an unknown combination of improvement in practice and reduction in performance with loss of motivation. Below we discuss genetic effects that may support this separation.

Table 1. Mean of median RT (msec), conflict scores (msec), and error rate by session of the ANT

| | Overall | | | |
|---|---|---|---|---|
| Session | Mean RT | SD | Error rate | Conflict score |
| 1 | 824 | 123 | .03 | 59 |
| 2 | 766 | 107 | .03 | 43 |
| 3 | 777 | 123 | .03 | 45 |

### Genetic effects

Table 2 shows the relation of the major behavioral findings to alleles of the three genes that we measured and hypothesized to be related to performance, DBH, MTHFR, and COMT. Some of the interactions between genes involve a small number of subjects in some cells; these are shown in Table 3. The effects described below show differences within subjects in repeated measures ANOVA analyses.

#### MTHFR X COMT

In accordance with our hypothesis, a repeated measures ANOVA including both COMT and MTHFR genotypes for the three sessions showed a main effect of MTHFR ($F(2, 124) = 6.59$, $MSE = 23528.26$, $p = .002$) and a significant interaction between MTHFR and COMT ($F(2, 124) = 5.72$, $MSE = 20410.02$, $p = .004$) on overall RT. As shown in Figure 2, the differential improvement in RT for the CC group with practice was only found for those children with the AA genotype of COMT.

However, reaction times were faster during Days 1 and 2 for the carriers of the T allele. The superior RT for those with the T allele was surprising, because a lowered level of methylation found with the T allele has been related to mental and physical illness (Roffman, Gollub, et al., 2008, Roffman, Weiss, et al.,2008) and poorer performance than those without a T allele (Hofstetter, Tavor, Tzur Moryosef, & Assaf, 2013). In support of this idea, the T group had slightly higher error rates on Days 1 and 2 than the CC group (see Table 2); this difference was not significant ($F(1, 64) = 1.22$, $MSE = 0.001$). The children with a T allele of MTHFR and AA genotype of COMT also had a slightly lower overall error rate than the other groups.

In agreement with the overall RT data shown in Figure 2, the ANT conflict scores show more

**Table 2.** ANT RT (msec) by session and genotype (number of participants)

| | Session | DBH GG | DBH GA/AA | MTHFR CC | MTHFR CT/TT | COMT AA | COMT AG/GG |
|---|---|---|---|---|---|---|---|
| RT | 1 | 814 (20) | 826 (48) | 841 (31) | 807 (37) | 802 (20) | 831 (48) |
| | 2 | 747 (20) | 773 (46) | 778 (31) | 755 (35) | 743 (20) | 775 (46) |
| | 3 | 787 (20) | 768 (46) | 765 (31) | 781 (35) | 736 (20) | 790 (46) |
| Error rate | 1 | .023 (20) | .030 (48) | .023 (31) | .032 (37) | .032 (20) | .026 (48) |
| | 2 | .027 (20) | .030 (46) | .027 (31) | .031 (35) | .028 (20) | .030 (46) |
| | 3 | .022 (20) | .029 (46) | .027 (31) | .027 (35) | .030 (20) | .026 (46) |

**Table 3.** Number of participants for gene X gene interaction

| | | COMT | | DBH | |
|---|---|---|---|---|---|
| | | AA | GA/GG | GG | AG/AA |
| MTHFR | CC | 9 | 21 | 9 | 21 |
| | TC/TT | 11 | 24 | 11 | 24 |

improvement over the three days for those homozygous for the allele supporting high methylation (C) of MTHFR when also homozygous for the A allele of COMT. However, overall better conflict resolution is shown by the T-present allelic group of MTHFR. This results in a significant interaction between MTHFR and COMT over the three sessions ($F(2, 124) = 4.57$, $MSE = 7537.75$, $p = .01$), similar to what is shown in Figure 2 for overall RT.

### Role of DBH

Another feature of the RT data was the overall longer RTs found on Day 3 than Day 2.

While overall the upswing was not significant, there was a significant difference between Sessions 2 and 3 RTs for the two allelic groups of DBH ($F(1, 64) = 4.74$, $MSE = 14272.94$, $p = .03$). As shown in Figure 3, the increase in RT from Days 2 to 3 occurred only with individuals homozygous for the G allele. There was also a significant interaction between DBH and COMT on the RTs between Days 2 and 3 ($F(1, 62) = 4.21$, $MSE = 11899.01$, $p = .04$) where a strong upswing in RT occurred only for the COMT AA group.

In addition, the strong practice effect from Day 1 to Day 2 produced an interaction between DBH and MTHFR on RT ($F(1, 62) = 5.77$, $MSE = 13482.99$, $p = .02$); the GG genotype of DBH showed a reduced practice effect when combined with the MTHFR high methylation allele (CC) but not otherwise. This suggests that the influence of waning attention may be found from the start of practice and not only during the upswing in RT on Day 3.

### HTKS

There was no main effect of MTHFR on HTKS score nor any interaction between MTHFR and COMT as found for ANT reaction time. However, there was a significant within-subjects effect between alleles of the COMT genotype and HTKS score, where the performance of AG/GG individuals declined significantly from Blocks 2 to 3 and that of the AA group remained high ($F(2, 130) = 3.79$, $MSE = 45.34$, $p = .03$). As shown in Figure 4, the AA group better maintained scores in the face of higher levels of conflict. In addition, there is a significant main effect of DBH ($F(1, 63) = 4.07$,

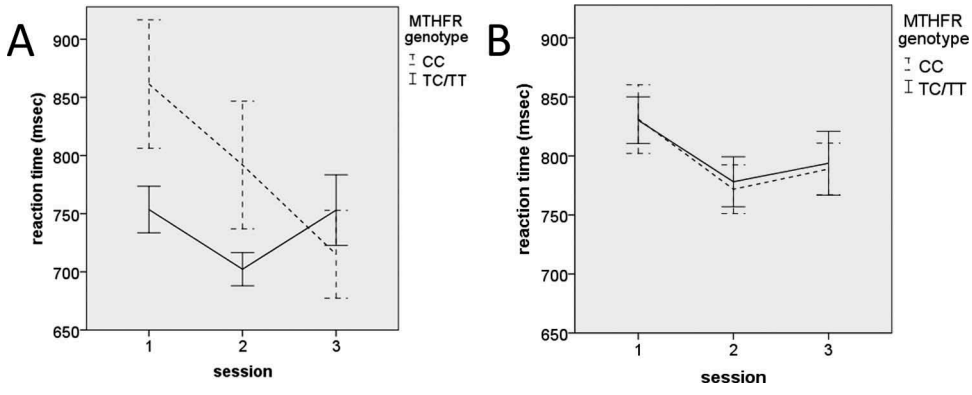


**Figure 2.** ANT overall RT by MTHFR x COMT genotype for each session of training for (A) COMT AA individuals and for (B) COMT AG/GG individuals. Error bars ± 1 standard error.

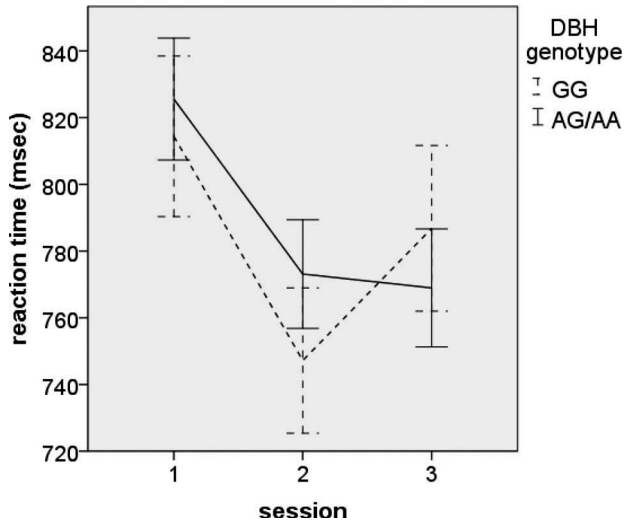


Figure 3. ANT overall RT by DBH genotype for each session. Error bars +/- 1 standard error.

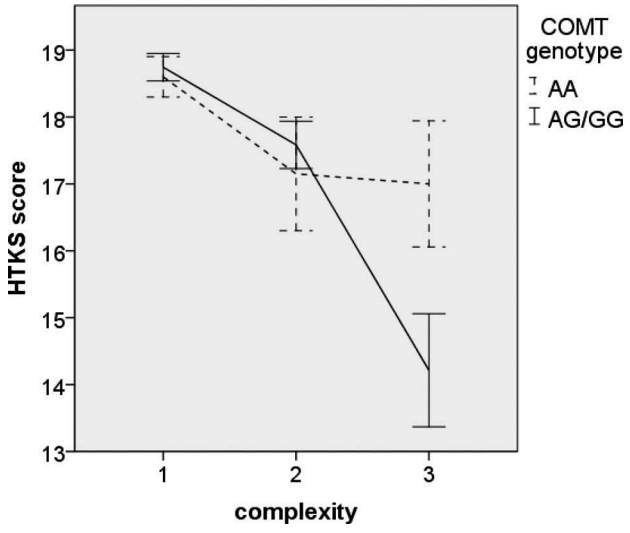


Figure 4. HTKS score by COMT genotype for each level of complexity (level 3 represents the highest conflict). Error bars +/- 1 standard error.

*MSE* = 64.21, $p$ = .048) and interaction between DBH and MTHFR ($F(1, 63) = 5.66$, *MSE* = 89.33, $p$ = .02) on the change in HTKS score between Blocks 2 and 3. The DBH GG individuals had a decreased score, and the MTHFR CC by DBH AG/AA individuals maintained their performance with complexity change, while those with other genotypes showed a decline.

## Discussion

### *Behavioral improvement*

There is remarkable agreement on practice-related decreases in reaction time. Tasks generally improve in reaction time and accuracy with practice (Fitts & Posner, 1967). For many skills, the extent of improvement slows down as practice continues, yielding a power function (Anderson, 1981; Anderson, Fincham, & Douglass, 1999; Fitts & Posner, 1967; Newell & Rosenbloom, 1981) or an exponential function (Heathcote, Brown, & Mewhort, 2000) with amount of practice. Regardless of the shape of the function, both forms suggest a single underlying process of improvement (Delaney, Reder, Staszewski, & Ritter, 1998). Newell and Rosenbloom (1981) proposed that a single process of chunking together responses was responsible for the improvement from the beginning of learning. In the ACT theory (Anderson et al., 1999), the power function is thought to emerge from a uniform increase in strength with repetition of procedures. Although some have argued that an exponential function fits better than the power function (Heathcote et al., 2000), there is general agreement on a monotonic function relating RT and practice.

In some cases, the improvement in reaction time may be followed by an increase in RT with further practice. The increase in RT with practice is often attributed to reduced motivation or attention as interest in the task declines. We have found this upswing in RT to be particularly strong in children (Kieras, 2006). Based largely on the performance of rats in mazes, Hull (1943) proposed that performance of a task might lead to the buildup of a reactive inhibition which would work to reduce habit strength and lead to a temporary increase in reaction time. Consistent with Hull's idea, we found a significant increase in RT from Day 2 to Day 3 for those children with the GG genotype of the DBH gene. A different polymorphism within the same gene had previously been found to be related to reduced attention in a task involving participants withholding responses to low-frequency stimuli. Differences in errors of commission were found between allelic groups (Greene, Bellgrove, Gill, & Robertson, 2009). It should be noted while both tasks required sustained attention, only our task examined the role of practice. In our study with adults (Voelker, Rothbart, & Posner, 2016), we found a similar tendency for the GG genotype. Although this finding is preliminary and requires replication with a larger sample, it is consistent with the Hull argument. The DBH GG genotype was also associated with a reduced practice effect from Day 1 to Day 2 suggesting that, like

reactive inhibition, it may be present even from the start of practice even though overall there is an improvement in RT.

### Individual differences in RT

Individuals differ in both the rate of improvement and in the likelihood of showing an increase in RT as practice continues. We have found that the improvement in RT in seven-year-old children is related to a gene that influences executive attention (COMT) in interaction with a gene that affects the efficiency of the process of methylation. We find that the CC genotype of MTHFR, which has been linked to better overall methylation (Llanos et al., 2015), shows improvement in RT over the three sessions. This learning effect occurs in interaction with COMT, suggesting that methylation works upon genes associated with cognitive performance. Similarly, the MTHFR CC genotype was associated with better HTKS performance under increased cognitive demand in interaction with DBH.

There have been several recent studies relating MTHFR and COMT to performance in cognitive tasks in normal adults and schizophrenic patients. In one study (Kontis et al., 2013), the MTHFR T allele reduced the negative effects of the AA version of COMT on performance and improved the performance of G carriers. On the other hand, Roffman and associates (Roffman, Gollub, et al., 2008, Roffman, Weiss, et al., 2008) showed that for those with the AA genotype of COMT, performance had more errors and longer RTs if they also had the T allele of MTHFR. In our view, such discrepancies may arise if participants are at very different levels of prior exposure to tasks and the findings confound learning in the task with their initial performance. In accord with theories of how epigenetic effects work (Day et al., 2013), we examined specific influences of genetic variation on learning with practice over three days.

### Methylation and behavior

In accordance with our hypothesis, individuals with the T mutation of MTHFR showed less improvement in RT over the sessions than those homozygous for the C allele. The T mutation presumably reduced learning by providing reduced opportunity for methylation. As shown in Figure 2, this effect was driven by the AA genotype of the COMT gene. Thus, children with low methylation efficiency and less-efficient dopamine degradation showed little evidence of improved performance with practice. We also examined a haplotype of the COMT gene related to high and low pain levels (Diatchenko, Nackley, et al., 2006; Diatchenko, Slade, et al., 2005), which we found to be related to performance during infancy (Voelker et al., 2009). In the current study, the genotype and haplotype showed similar results so we reported only the genotype in this paper.

However, the clear advantage of the MTHFR CC genotype in the presence of the AA genotype of COMT for learning is reversed if one looks at RT performance on Day 1 alone. In this case, the CC genotype is much worse overall than for those children who have a T mutation present and are in the COMT AA allelic group (see Figure 2). The lack of a practice effect when the T allele is present might be due to a floor effect on RT. However, in Table 2, RTs for other groups are faster than for the T-present group on Day 1 and all groups show improvement on Day 2.

Another possible explanation for the faster RTs for children with the T allele may occur because of a tendency toward impulsivity, since it has been reported that children who have the T mutation have elevated levels of ADHD (Gokcen, Kocak, & Pekgor, 2011). Children with ADHD often show impulsivity as a trait. The somewhat higher error rates for carriers of the T allele on Days 1 and 2 (see Table 2) provide some support. However, those children with the MTHFR T allele and the COMT AA genotype who showed fast RTs also show a slightly lower overall error rate. Their combination of fast RTs with reduced error is clearly inconsistent with the general impulsivity of those with the T allele.

Studies showing poorer performance of participants with the T allele involve adults. They generally showed longer RTs and more errors than participants with the CC genotype. (Kamdar et al., 2011; Roffman, Gollub, et al., 2008; Roffman, Nitenson, et al., 2011; Roffman, Weiss, et al., 2008). A population of childhood leukemia survivors showed differences in attention and processing speed by MTHFR genotype (Kamdar et al., 2011). It is possible that the difference in age between our study and other studies may account for the advantage of those with the T allele in overall reaction time. In summary, children with

the T allele show better overall performance but less improvement with practice, while adults with the same allele show overall poorer performance.

### *Attention and persistence*

A second feature of the ANT data was the upswing in RT between Days 2 and 3.

It is common for children to show a performance to peak at some time and then to show a reduction, probably due to reduced attention and motivation (Kieras, 2006). The DBH GG genotype shows a significant increase in RT between Day 2 and Day 3. The GG genotype was also associated with a decline in performance on the HTKS with the increased challenge that occurred on Day 3. Since difficulty of the task and amount of practice was confounded in this study, we cannot say which was associated with the reduced performance of the GG genotype. Other studies have implicated polymorphisms in this gene with the lack of persistence during RT tasks (Bellgrove, Hawi, Gill, & Robertson, 2006; Greene et al., 2009). Both the promoter polymorphism of Greene et al. (rs1611115) and the exon 2 synonymous variation investigated in this work (rs1108580) have been associated with significant differences in transcriptional expression of the gene (Barrie et al., 2014). The expression of DBH is tissue-dependent, while measurements in the liver showed clear allelic differences, that of the locus coeruleus remained non- significant, possibly due to technical limitations. Previously, we found the GG polymorphism is most related to slower responding when no cue is given, a condition that has been associated with lower tonic alertness (Posner, 2008). Since the brain mechanisms of tonic alertness have been associated with the norepinephrine system in the locus coeruleus, studies linking motivation to continue the task and attention networks might be useful in understanding the neural basis of motivation.

The finding of no significant correlation between the improvement in RT from Day 1 to Day 2 and the increase in RT between Days 2 and 3 provides some support for separating these two features of practice based on their opposite effect on overall RT. However, it seems unlikely that improved performance with practice and diminished performance with reduced motivation are occurring at completely separate times. In one common theory of learning, effective performance at any time is a combination of habit strength from practice and reactive inhibition based on repeated trials (Hull, 1943). Our finding of an influence of DBH in conjunction with MTHFR on improved performance (Day 1 to Day 2), as well as the upswing in RT (Day 2 to Day 3), generally supports the idea of both faster RT due to practice and slower RT due to waning attention throughout practice and suggests that no single underlying factor accounts for the power function often found in RT with number of trials.

### *Mechanisms of change*

Fjell and colleagues (Fjell et al., 2012) have shown that reaction time of children and young adults in the flanker task depends heavily on the functional connectivity between the ACC and other areas. Recent work in mice shows that learning motor skills depends on the activation of oligodendrocytes leading to improved myelination (McKenzie et al., 2014). A recent study in rats demonstrated changes in gene expression related to gene methylation status in the ventral tegmental area (VTA) during reward-related learning. Learning was inhibited in the presence of a DNA methyltransferase inhibitor (Day et al., 2013). Hypermethylation within the gene body was shown to be associated with increased gene expression in a neuronal activity-dependent manner. This hypermethylation was required for learning and not subsequent memory retrieval. MTHFR may be playing a key role in facilitating this learning-based mechanism and one possibility is that it may be regulating COMT and other genes in the dopaminergic pathway in a similar manner to that shown for plasticity genes of the VTA. Thus, more efficient MTHFR activity may support learning by facilitating gene body methylation in genes relevant to the learning process.

Why is MTHFR working for COMT AA and not for COMT G carriers? Individuals homozygous for the lower activity allele (AA) have shown better cognitive performance, presumably because higher synaptic DA levels would allow greater opportunity for DA signaling and thus enhance neuronal activity. If learning requires gene methylation in an activity-dependent manner, and COMT AA individuals have more activity, there would be more potential for gene modification. This, combined with MTHFR CC, makes it possible for maximal gene methylation to occur and support the strongest response to

learning. This mechanism would suggest that MTHFR and COMT work in concert to provide the optimal environment for learning-related gene regulation, and would not necessarily implicate MTHFR in the modification of COMT expression.

Our study shows important differences between MTHFR groups in the effectiveness of practice on improving reaction time within two sessions. It appears that the efficiency of white matter may be changed rapidly by spatial training (Hofstetter et al., 2013), working memory (Takeuchi et al., 2010), or meditation (Tang et al., 2010). The role of glia in the production of myelin is well-documented and activated axons transmit signals to neighboring glial cells, thereby promoting myelination (Hofstetter et al., 2013). Studies in mice show clearly that potentiation of ogliodendrocytes is one necessary condition for skill learning (McKenzie et al., 2014). We have hypothesized that a similar mechanism may operate in improved white matter following brief meditation training in humans (Posner, Tang, & Lynch, 2014). These studies suggest that improved reaction time with practice found in our study could arise by improving the efficiency of white matter connections between the ACC and motor regions.

The one-carbon folate cycle, in which MTHFR plays a major role, is tightly regulated and supports many crucial processes that play a role in learning, including neurotransmitter function and epigenetic regulation. Changes in DNA methylation coincide with the maturation of neural progenitors and methylation factors have been shown to control the timing of astrogliogenesis (Fan, Martinowich, et al., 2005; Teter et al., 1996). Diseases resulting in demyelination, such as Alzheimer's disease and multiple sclerosis, show differences in DNA methylation patterns in the brain (Bakulski et al., 2012; Huynh et al., 2014). We propose that MTHFR activity influences individual differences associated with DA signaling through changes in the expression of genes that support learning, and that these changes ultimately result in possible differences in neural myelination. Since the one-carbon folate cycle influences many cellular functions, future research should address the specific mechanism(s) of methylation responsible for differences in learning.

### *Relation to plasticity*

This Special Issue is about brain plasticity. We believe that an important mechanism of plasticity is in the efficiency of white matter connections between brain areas involved in the task. Many studies using animal models or Diffusion Tensor Imaging (DTI) as indicants have found that various forms of learning can affect the efficiency of white matter pathways between brain areas involved in the task (see Wang & Young, 2014; Zatorre, Fields, & Johansen-Berg, 2012 for reviews). For example, we have used DTI to show that fractional anisotropy, a parameter related to connectivity, can be changed following a month of meditation training (Tang et al., 2010). We have speculated that this change may be induced by the increased frontal theta rhythm found following such training (Posner et al., 2014). This role for theta would be built upon the finding that Long-Term Potentiation (LTP), a major brain mechanism related to learning, can be induced in hippocampal cells following theta stimulation (Larson, Wong, & Lynch, 1986). Repeated stimulation of a broad population of cells via theta might influence their connectivity.

There is a lot of evidence that methylation plays a role in plasticity in animal models (see Day et al., 2013 for a review). However, ties between epigenetic mechanisms such as methylation and white matter changes that occur during human skill learning (Wang & Young, 2014) have not, to our knowledge, been made previously. Much fMRI work has shown that various forms of learning can change functional connectivity between nodes involved in a relevant network. Our studies, while preliminary, attempt to provide a possible genetic mechanism by which the extent of white matter change found in learning might differ among individuals.

We believe our findings indicate that practice on a task involves both improvements in reaction time due in part to improved myelination of relevant pathways and decrements in performance due to lowered levels of alertness to the task. Individual differences in these practice effects may be due to the efficiency of epigenetic methylation leading to differences in the rate at which practice changes performance. Future studies will be needed to examine the generality of these findings to different ages and types of performance.

### *Cautions*

Our study is an exploratory one. The sample size overall is small and this is exaggerated for the gene X gene interactions. While the MTHFR predictions

were made in advance of the study, those for DBH arose from our genotyping of potential genes that influence sustained attention and must be considered as tentative. For this reason, we have repeated and extended the study with 70 adult subjects performing the ANT and two other tasks. In that research (Voelker et al., 2016), we have replicated an interaction between MTHFR and COMT on the ANT conflict network and extended the methylation finding to a serial reaction-time task.

There are several mechanisms by which improved methylation might influence performance. Some of these involve modification of the gene by adding a methyl group, while others are direct effects of protein methylation, which facilitate the formation and stability of the myelin sheath (Kim, Lim, Park, & Paik, 1997) or phospholipid methylation influencing neural membrane fluidity (Sharma et al., 1999), possibly resulting in differences of neural synchrony (Kuznetsova & Deth, 2008). Additionally, the MTHFR 677T variation has been associated with higher plasma homocysteine levels (Colson, Naug, Nikbakht, Zhang, & McCormack, 2015), and high homocysteine levels have been associated with poorer cognitive function in elderly populations (Kong et al., 2013; Ravaglia et al., 2003). However, less is known about the relationship between cognitive function in children and homocysteine levels. In a cohort of children with epilepsy, high homocysteine levels were associated with the 677T allele, but not with IQ (Di Rosa et al., 2013). Short-term supplementation of folate in children with low folate levels decreased homocysteine levels, but did not influence cognitive performance (Rauh-Pfeiffer et al., 2014). In order to know if the MTHFR effect actually involved modulation of the genome, it would have been useful to show that our training influenced the methylation of candidate genes involved in learning, or to use a whole genome measure of methylation in brain areas related to the anterior cingulate. Since we used saliva, no analysis of brain areas was possible and we only collected saliva from each participant prior to training, so we could not examine differences in methylation due to training, even if we assumed similarities in the epigenetic modulation of genes in brain and saliva. Moreover, our current methods do not allow elimination of the possibility that MTHFR works via a different mechanism than modulation of the genome or that a correlated genetic influence might be responsible for these effects. Animal studies may be able to show more directly the exact mechanism involved in these findings. In experiments we have currently underway with mice, we hope to address these issues.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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