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Involvement of the AATn polymorphism of the *CNR1* gene in the efficiency of procedural learning in humans

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ABSTRACT

Procedural learning refers to the acquisition of motor skills and the practice that refines their performance. The striatum participates in this learning through a function regulated by endocannabinoid signaling and other systems. This study relates the efficiency in learning a procedural task with the AATn polymorphism of the *CNR1* gene, which encodes for the CB1 receptor. The mirror-drawing star task was solved by 99 healthy young subjects in three trials. The sample was divided into high- and low-performance groups based on performance efficiency. AAT12/14 carriers were more frequent in the former group, while there were more AAT12/13 carriers in the latter, which also made more errors/min. Therefore, we characterized two efficiency phenotypes: high- vs. low-performers associated with the two AATn genotypes, AAT12/14 vs. AAT12/13. The findings suggest that AATn polymorphism modifies CNR1 translation, indicating a different modulation of CB1.

In everyday life we use many motor sequences (driving, dancing, writing), skills that gradually improve with practice once acquired [14,12,1]. Motor sequence learning depends on the activity of the striatum, the motor and premotor cortices, the supplementary motor area, the hippocampus and the cerebellum [12,1]. The striatum increases its activation as performance improves [1,17,35] and through the successful execution of a previously acquired sequence [26,32]. Thus it plays a crucial role in acquiring procedural learning and forming procedural memory [9,18,31,27,39].

The activity of the dorsal striatum depends on a complex neurochemistry. The striatal GABAergic medium spiny neurons (MSN), the main type found in the striatum, are regulated by glutamatergic and dopaminergic afferents sent by other brain structures. Locally, the MSN are regulated by GABAergic and cholinergic interneurons. The MSN then self-regulate their excitability by producing retrograde messengers, such as endocannabinoids. Complementary glutamate and GABA terminals express high concentrations of the cannabinergic receptor 1 (CB1) [36,20,6]. Studies have shown that administering cannabinoids to the dorsal striatum of rats modifies the strategy they employ to solve the Barnes Maze [30]. Also, through the CB1 receptor, the cannabinoid system participates in extinguishing procedural memories [31].

The human CNR1 gene (6q14–q15) codes for CB1. The 3′ extreme of this gene has a polymorphic AATn triplet [40]. Although there is no evidence of its functional role, this type of microsatellite localized at 3′ UTR could affect gene translation [11]. Several studies relate the AATn polymorphism of *CNR1* to disorders such as schizophrenia [37,2,21,33], addictions [40,2,8,16,10,29], impulsivity [13], depression and Parkinson's disease [3]. Differences in AATn have been reported between patients and controls. Given that this polymorphism is part of the human genome, but not an exclusive marker of pathology, AATn polymorphism may be related to cognitive function efficiency in healthy subjects. The association of AATn polymorphism with cognition has been explored in relation to attention only in subjects with some medical condition. Drug-

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dependent Caucasian subjects carrying at least one AAT < 5 allele exhibited an increased P300 amplitude of the event-related potentials, suggesting a higher attention capacity than homozygotes for AAT ≥ 5 alleles [16]. Also, alcoholic Spanish patients who suffered attention deficit/hyperactivity disorder during childhood have at least one AAT ≥ 5 repeat allele [29].

We had two objectives in examining the AATn polymorphism of CNR1: first to describe the allelic and genotypic distributions of AATn repeats in healthy Mexican subjects; second, to do an exploratory study of this polymorphism's role (no *a priori* assumptions were made) in procedural learning using the mirror-tracing star task [22], which easily evaluates motor skill learning in only a few trials. Procedural learning tasks depend on the striatum, which activates during both learning [1,17,35] and the extinction of procedural memories [31], where CB1 is highly expressed. We hypothesized that if this polymorphism participates in CNR1 translation it might be reflected in the efficiency in learning a procedural task. First, we examined AAT ≥ 5 and <5 in our sample to see if these alleles are present in healthy Mexican subjects. Next, we compared AATn frequency as a function of behavioral efficiency, and tested to determine if this polymorphism was specific to procedural learning, or was also related to recognition memory.

Participants. Ninety-nine (50 males), young, right-handed (mean ± SD: 23.71 ± 2.07 years old; 16.34 ± 1.90 years of schooling; normal-to-corrected vision), neurologically and psychiatrically healthy subjects (Edinburgh Inventory [25]) were recruited. Beck's Depression and Anxiety Inventories were used to eliminate prospective participants with depression or symptoms of anxiety just before the experimental session. Subjects were taking no medications that might have affected their central nervous systems during the session, nor did participants have histories of drug addiction, and none reported having consumed illicit drugs. All answered the Wechsler Adult Intelligence Scale-Vocabulary subscale and the U.S. National Sleep Foundation Register (to ensure that vocabulary use and sleep hours did not differ among genotypes). Experiments were performed following one of three schedules: 10, 13 or 16 h, to keep diurnal effects constant [23]. Our study was approved by the Faculty of Medicine's Research and Ethics Committee (UNAM). After receiving a complete description of the research design, subjects signed an informed consent form.

The mirror-tracing task. Participants had to trace a third inner star in a clockwise direction inside a five-pointed, double-margin star (5 mm between borders) on a letter-size sheet of paper. They could not look at the drawing directly, only its reflection in a mirror. Subjects performed three consecutive trials, beginning at the upper peak and ending when the star was complete. No time limit was set for solving the task, but duration was recorded. This test was selected because it is a new visual-motor skill for subjects, which is learned only after several trials [22]. The variables quantified per trial were Time for Tracing and Number of Errors (average from five referees: number of times borders were touched plus the times subjects lifted the pencil). A Performance Index (PI) was calculated to relate Time of Tracing to Number of Errors. It allowed us to estimate the number of errors/min of performance. PI was calculated by dividing the number of errors in one trial by the tracing time required and multiplying by 60 (s).

Assessment of recognition memory. This procedure involved a computerized task. Each trial presented a dark gray asterisk – fixation point – against a light gray background for 1000 ms. Participants had to encode 20 faces (i.e., classify them as female or male) by pressing a button (horizontal visual angle, HVA: 3.15°, vertical visual angle, VVA: 3.44°), and then encode 20 scenes (i.e., classify them as indoors/outdoors; same visual angles), also by pressing a button. All images appeared in the center of a monitor. Immediately afterwards, subjects performed a recognition task: 40 previously encoded stimuli were presented randomly interspersed with 40 new ones and they had to decide whether each stimulus was new or had appeared in the earlier phase. In both phases (encoding, recognition) images lasted 800 ms and response time was 2000 ms. Recognition probability (i.e., likelihood of correct responses for old stimuli minus likelihood of incorrect responses for new stimuli) was analyzed.

Assessment of motor function. Participants were evaluated on a simple reaction-time task. One capital letter (HVA: 0.45°, VVA: 0.69°) was shown in the center of a monitor. Each letter appeared for 500 ms followed by an inter-trial interval of 508-to-1492 ms (mean = 1000 ms), to prevent habituation. Subjects pressed a button on a response box as soon as they detected the letter's presence on the screen; response time was limited to 1000 ms. Two blocks of 120 trials were conducted. Participants responded by pressing a button with their right or left index finger (60 trials/finger). Response order was counterbalanced among subjects. Percentages of correct responses and reaction times were analyzed.

Genotyping. Saliva samples were collected using the Oragene DNA Self-Collection Kit (DNA Genotek Inc., Kanata, Ontario, Canada). Deoxyribonucleic acid (DNA) was isolated following the Oragene DNA Purification Protocol. The amount of DNA extracted was quantified by absorbance spectroscopy (ND-1000 NanoDrop Spectrophotometer) and diluted for working solutions to 50 ng/μL. The DNA isolated and the working solutions were stored at 4 °C. PCR reactions were performed in a 50 μL reaction volume containing 200 ng of genomic DNA, 0.5 μM of each primer, 0.2 mM of each deoxynucleotide triphosphate, 1× PCR buffer, 1.5 mM $MgCl_2$, and 1 U AmpliTaq gold polymerase (Applied Biosystems, Foster City, CA, USA). The AATn polymorphism was sequenced after amplification by PCR with forward (5′-TAC-ATC-TCC-GTG-TGA-TGT-TCC-3′) and reverse (5′-GCT-GCT-TCT-GTT-AAC-CCT-GC-3′) primers. Steps for the PCR conditions were 95 °C for 10 min, 30 cycles at 95 °C for 45 s, 56 °C for 45 s, 72 °C for 1 min and an elongation step at 72 °C for 7 min. PCR products were verified in a 1.5% agarose gel and photographed before DNA sequencing. Products were then cleaned with the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA). Automated DNA sequencing was done on an ABI 3730XL DNA Analyzer sequencer using BigDye Terminator Cycle Sequencing Kit version 3.1 reactions (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed with Lasergene v7.0.0 (DNASTAR, Inc., Madison, WI, USA.). Also, AATn polymorphism was detected after amplification by PCR with forward (5′-TAC-ATC-TCC-GTG-TGA-TGT-TCC-3′), labeled with 6-FAM (Applied Biosystems, Foster City, CA, USA), and reverse primers (5′-GCT-GCT-TCT-GTT-AAC-CCT-GC-3′). Samples were run on the ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), while genotyping results were analyzed using GeneMapper software V3.7 (Applied Biosystems, Foster City, CA, USA). Probes and oligonucleotides were obtained from Applied Biosystems using the Assay-on-Demand product. PCR amplification was performed using the 7900 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. As a laboratory quality control measure, fragment analyses were used for all DNA samples to detect the AAT microsatellite and the number of repeats of each subject was validated by sequencing. The concordance rate reached 100%.

Statistical analysis. The Hardy–Weinberg equilibrium was calculated using Arlequin V3.11 software (http://cmpg.unibe.ch/software/arlequin3). Chi-squares (χ^2) were calculated for the allelic and genotypic distributions from the entire sample. The allelic distribution of this polymorphism was described because comparisons among populations of different ethnic origin are crucial to understanding the inter-individual variability of the CNR1 expression. On the other hand, we hypothesized that describing the genotypic distribution might allow us to see if a specific genotype had an effect on procedural learning. The alleles and genotypes that

Table 1
Allele frequencies and genotype distribution (percentages shown in brackets) of the AATn polymorphism of CNR1 in the entire sample, and as a function of performance in the procedural learning task.

	Allele frequencies[a]: # (%)								*p*-Values
	7	9	10	11	12	13	14	15	
Complete sample	5(2.53)	2(1.01)	25(12.63)	9(4.55)	54(27.27)	38(19.19)	64(32.32)	1(0.51)	<0.00001
High performance group	2(2.00)	2(2.00)	14(14.00)	3(3.00)	27(27.00)	15(15.00)	36(36.00)	1(1.00)	0.32[a]
Low performance group	3(3.06)	0(0.00)	11(11.22)	6(6.12)	27(27.55)	23(23.47)	28(28.57)	0(0.00)	

	Genotype distribution: # (%)										*p*-Values
	7,10	7,12	7,14	9,12	9,13	10,10	10,11	10,12	10,13	10,14	
Complete sample	1(1.01)	3(3.03)	1(1.01)	1(1.01)	1(1.01)	1(1.01)	1(1.01)	10(10.10)	5(5.05)	6(6.06)	<0.001
High performance group	1(2.00)	1(2.00)	0(0.00)	1(2.00)	1(2.00)	1(2.00)	0(0.00)	5(10.00)	2(4.00)	4(8.00)	0.005[b]
Low performance group	0(0.00)	2(4.08)	1(2.04)	0(0.00)	0(0.00)	0(0.00)	1(2.04)	5(10.20)	3(6.12)	2(4.08)	

	Genotype distribution: # (%)									
	11,12	11,13	11,14	12,12	12,13	12,14	12,15	13,13	13,14	14,14
Complete sample	2(2.02)	1(1.01)	5(5.05)	3(3.03)	12(12.12)	19(19.19)	1(1.01)	5(6.06)	7(7.07)	13(13.13)
High performance group	1(2.00)	0(0.00)	2(4.00)	1(2.00)	2 (4.00)[c]	14 (28.00)[c]	1(2.00)	4(8.00)	2(4.00)	7(14.00)
Low performance group	1(2.04)	1(2.04)	3(6.12)	2(4.08)	10(20.41)	5(10.21)	0(0.00)	2(4.08)	5(10.20)	6(12.25)

[a] The alleles and genotypes that did not attain a frequency at least of 5 were excluded from the analyses.
[b] *p*-Values for the CLUMP T2 test [34] for comparing the high-performance and low-performance groups.
[c] Genotype frequency differences between the high- and low-performance groups, Yates' $\chi^2 = 7.43$, $p = 0.006$.

did not attain a frequency of five were excluded. CLUMP software [34] was used to compare the genotypic and allelic distributions between the high- and low-performance groups. Significance was assessed using a Monte Carlo approach (number of tables with higher χ^2 value in 1 simulation of contingent tables with the same marginal totals as the observed distribution). To avoid violating the χ^2 test for small cases (<5), T2 statistics from the CLUMP software were used. T2 is employed when expected values are <5, then its column is added to that with the next smallest total until reaching a value of at least five [34]. To compare differences between the AAT12/13 and AAT12/14 frequencies, the Yates' χ^2 test was used. Dependent variables from the two performance groups (group factor: high-performance $n = 50$; low-performance, $n = 49$) or the two genotypes (AAT12/13, $n = 12$; AAT12/14, $n = 19$), were compared using mixed Variance Analysis. Trial (1, 2 and 3) was the intra-subjects factor. Bonferroni was used as a *post hoc* test. Analyses included control of the False Discovery Rate (FDR) for multiple comparisons at a significance threshold of $p < 0.017$ [4,5].

We observed eight alleles in this Mexican sample, from AAT7 to AT15 but without AAT8 (Table 1). A significant difference in allelic distribution emerged ($\chi^2_5 = 87.68$, $p < 0.0001$). The AAT14 and AAT12 alleles were the most frequent ones in this sample, and both differed in frequency from the other six repeat alleles ($p < 0.001$). Moreover, our sample showed a difference in genotypic distribution ($\chi^2_8 = 20.51$, $p = 0.009$). The AAT12/14 genotype was more frequent than all others (Table 1). AATn repeats were in the Hardy–Weinberg equilibrium ($p = 0.41$).

We were unable to divide our sample as in earlier studies (i.e., <5 vs. ≥5 [8,16]) as no subjects had AAT < 7, so we probed whether some AATn polymorphism might be related to efficiency in the procedural task by splitting the total sample as a function of PI (errors/min; integrating Time and Number of Errors during Tracing) into high- ($n = 50$) and low-performance ($n = 49$) groups. The median was 9.75 errors/min (range: 0.05–47.06; see Table 1: Supplementary material). This allowed us to detect whether one particular AATn genotype appeared more often in one performance group (differences not explained by other variables, see Table 1: Supplementary material). Therefore, the two genotypes AAT12/13 and AAT12/14 (Table 1) were significantly different between the high- and low-performance groups (CLUMP T2: $\chi^2_2 = 10.55$, $p = 0.005$). A 2X2 crosstab performance by genotype performed using Yates' correction ($\chi^2 = 7.43$, $p = 0.006$) revealed that the AAT12/14 genotype was statistically more frequent in the high-performance group ($\chi^2_1 = 4.26$, $p = 0.04$), while the AAT12/13 genotype was more frequent among low achievers ($\chi^2_1 = 5.33$, $p = 0.02$).

To detect if one of these AATn genotypes of CNR1 had an effect on performance of the procedural learning task, data was analyzed according to the AAT12/13 ($n = 12$) vs. AAT12/14 ($n = 19$) genotypes (the most frequent ones in our sample) regardless of performance group. We observed a statistical difference only between genotypes, using control of FDR for multiple comparisons in the PI ($F_{1,29} = 6.72$, $p = 0.015$). The AAT12/14 carriers made fewer errors/min (9.00 ± 2.18) than the AAT12/13 carriers (18.07 ± 2.74). Table 2 shows descriptive results according to the AAT12/13 and AAT12/14 alleles. Also, we analyzed performance as a function of AAT 12, 13 and 14, but detected no effect ($p > 0.05$).

To test whether these differences between genotypes were specific to procedural learning, we assessed recognition memory (part of declarative memory), but no differences appeared ($p = 0.59$, Table 2). Likewise, to discover if there were differences in motor function between genotypes, the percentage of correct responses and reaction times from the simple reaction time task were analyzed; again, no effect was found ($p = 0.70$ and $p = 0.13$, respectively; Table 2).

Two important findings emerge from this study. First, we describe the AATn allelic and genotypic distribution in healthy young individuals from central Mexico; second, we report the involvement of AATn polymorphism in procedural learning.

The AAT14 allele was the most frequent one (32.32%), followed by AAT12 (27.27%). Together they represented 60% of the sample. Eight AATn alleles were present: AAT7 and AAT9–AAT15. The AATn allelic and genotypic distributions observed are different from those described for normal healthy control subjects in other populations; e.g., AAT4 (36.4%) was reported for a Californian-Caucasian [8] and an African-American [11] sample; AAT8 (30.1%) in a European-American sample [10]; and AAT10 (30.7%) in a German one. Larger alleles, like AAT12, were described in European-American (36%) and African-American populations (38.5%), while AAT15 was found in Japanese controls (34.5%; similar to [37]), and AAT13 (37.5%) in a sample from Martinique [2].

Table 2
Descriptive data and results from the procedural learning, recognition memory and simple reaction time tasks as a function of the AAT genotype groups 12/13 and 12/14. The False Discovery Rate for multiple comparisons was corrected using a significance threshold of $p < 0.017$ [4,5]. Statistical differences are marked with *.

AAT genotypes	12/13	12/14	*p*
N	12	19	
Male/female (#)	7/5	11/8	0.58
Age (mean ± SD)	23.58 ± 2.54	25.05 ± 2.92	0.16
Years of schooling (mean ± SD)	16.75 ± 1.56	15.93 ± 1.85	0.22
Edinburgh Handedness Inventory (mean ± SD)	88.55 ± 13.10	88.32 ± 14.59	0.96
Wechsler Adult Intelligence Scale-Vocabulary subscale	45.05 ± 10.57	52.58 ± 12.12	0.08
Sleep hours the week before the experimental session (mean ± SD)[a]	7.49 ± 1.12	7.55 ± 1.32	0.90
Sleep hours the night before the experimental session (mean ± SD)[a]	7.56 ± 1.41	7.07 ± 1.56	0.38
Procedural learning (mean ± SE)			
Errors (#)	37.08 ± 7.40	22.11 ± 5.88	0.08
Time of drawing (ms)	148.10 ± 26.10	154.67 ± 20.74	0.85
Performance index (errors/min)	9.00 ± 2.18	18.07 ± 2.74	0.015*
Recognition memory			
Probability of recognition	0.44 ± 0.06	0.41 ± 0.05	0.59
Simple reaction time task			
Correct responses (%)	99.38 ± 3.16	97.41 ± 2.51	0.70
Reaction time (ms)	320.08 ± 13.65	336.31 ± 10.85	0.13

[a] Sleep hours the week before and the night before the experimental session were reported by subjects through the National Sleep Foundation of the United States of America diary.

In contrast, we found 20 different AATn genotypes (Table 1). AAT12/14 (19.2%) was the most frequent one in our sample. In comparison, AAT13/14 (17.0%) was the most frequent genotype in a control sample from Martinique [2]. These differences in the allelic and genotypic distributions suggest that the AATn repeats in the *CNR1* gene are heterogeneous among populations and that the Mexican sample had ancestral contribution patterns distinct from those of pre-Columbian natives and the later European colonists [38].

Our second important finding was the association of the AATn polymorphism of the CNR1 gene with efficiency in resolving the mirror-tracing task. The entire sample revealed similar learning results to those found by others with respect to the number of errors [22,28,15] and time required for tracing [15], changes that suggest that our subjects did indeed learn this new visual-motor skill [15].

We could not divide our sample as a function of AAT5 as other authors have done [8,16,29,3], due to the absence of AAT < 7 in our sample. It may be that AAT < 7 is more frequent in other, non-Caucasian populations, or may be related to a greater propensity to addiction [8,16,29]. For these reasons, we divided our sample into high- and low- groups as a function of performance. The groups were homogeneous in age, education, and the number of hours of sleep per week (supplementary material, Table 1), differing only in the frequency of AAT12/13 and AAT12/14 carriers (Table 1). The AAT12/13 carriers made twice as many errors/min as the AAT12/14 carriers (Table 2). Therefore, being a carrier of at least one of the AAT12, AAT13, or AAT14 genotypes is insufficient to make a difference in skill performance, as this phenotype difference arose only for AAT12/13 and AAT12/14. The effect of the AATn genotype was restricted to procedural learning; neither recognition memory nor motor function was associated with AATn polymorphisms. No effect can be attributed exclusively to motor function, only to the learning process involving the striatum which might, therefore, demand activation of CB1 [31]. Hence, the *CNR1* gene contributes to procedural learning, a multifactorial trait. Other genotypes may also participate in explaining the variability observed in the way of learning a visual-motor skill; for example, dopaminergic DRD2 polymorphism due to the high distribution of DR2 in the striatum [19], or the interaction of the *CNR1* and DRD2 genes. The task evaluated here depends on the striatum, which is highly activated during procedural learning [1,17,35,31]. In its basal state, the CB1 receptor is highly expressed in the striatum [36,20,6] and its role in long-term synaptic plasticity in this structure has been described [19]. Thus, the role of CB1 in the striatum that modulates procedural learning might vary as a function of the AATn polymorphisms in the CNR1 gene. To date, the functional role of AATn polymorphisms remains unclear, but it has been suggested that microsatellites, such as AATn, could form the Z-DNA structure, which modifies gene translation [29,3], and that this could be a function of the microsatellite's length [7]. However, the physiological differences between AAT12/13 and AAT12/14 carriers remain unclear in terms of expression, distribution and the binding of eCBs to the CB1 receptor. One might speculate that the CB1 receptor inhibits the striatum more strongly in AAT12/14 carriers than in AAT12/13 carriers, thus allowing greater control in the coordination of movement.

Some limitations of our study must be addressed. First, the level of expression of the CB1 receptor in the brain is unknown, though it is crucial to describing the functional relation of AATn polymorphism in the CNR1 gene and the expression of CB1. Second, the number of subjects was small for a genetic association study; however, there was no deviation from the Hardy–Weinberg equilibrium [24]. It is important to note that this is an exploratory study of CNR1 and a cognitive trait. However, the sample size had a power of 0.49 to detect an effect size of 0.84 between AAT12/13 and AAT12/14 at $\alpha < 0.017$. Although the power is lower than 0.8, the exploratory character of this study allows us to suggest the potential involvement of CNR1 in procedural learning.

In conclusion, we described the most frequent allele (14) and genotype (12/14) of the AATn polymorphism of the *CNR1* in a sample of healthy Mexican adults. The AAT12/13 genotype was related to a lower efficiency (more errors/min) phenotype in a procedural learning task, compared to the AAT12/14 genotype. These results support the idea that AATn polymorphism modifies CNR1 translation, and suggest a different modulation of CB1. This association is specific to procedural learning but not to recognition memory or motor function.

Conflict of interest

None of the authors have any conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2011.03.013.

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