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Key words: *Hyperactivity, melatonin, circadian rhythm, sleep, Clock*

Running title: *The melatonin pathway in ADHD*

Abstract

Sleep wake cycles are frequently disturbed in patients with Attention Deficit and Hyperactivity Disorders. We hypothesized that the origin of the sleep problems may be the consequence of an abnormal circadian clock setting regulated by the melatonin pathway. Here, we sequenced all the genes of the melatonin pathway *AA-NAT*, *ASMT*, *MTNR1A*, *MTNR1B* and *GPR50* in 328 individuals from Sweden including 108 patients with ADHD and 220 from the general population. Non-synonymous mutations were identified in all genes at a similar frequency in patients with ADHD and in controls. Among the functional variations, a splice site mutation (IVS5+2T>C) in *ASMT* and one stop mutation (Y170X) in *MTNR1A* were only detected in patients with ADHD. Biochemical analyses indicated that these mutations abolish the activity of ASMT and MTNR1A. We also identified clusters of SNPs within *MTNR1B* showing significant difference in the allelic frequency between ADHD patients and control (maximum signal at rs10830961 *P*=0.0002). Taken together, these genetic and functional results shed light on one new compelling candidate pathway for susceptibility to circadian rhythms alterations that could help clinicians for providing better treatments of patients with ADHD and sleep problems.

Introduction

Attention Deficit/ Hyperactivity disorder (ADHD) is one of the most common pediatric psychiatric disorder, estimated to occur in 3% to 7% of school-age children [1]. Alteration of sleep is frequently observed in patients with ADHD [2,3]. The most common sleep disorder associated with ADHD is initial insomnia[4]. Sleep problems were originally regarded as a consequence of ADHD, but they may actually be primary risk factors for ADHD. Indeed, it is now well established that mutations altering the biological clock can cause severe sleep problems by modifying sleep phase or duration[5-7]. Furthermore, several studies have found delayed dim light melatonin onset (DLMO) in patients with ADHD and chronic sleep onset insomnia[4]. Finally, melatonin was shown to greatly improve the sleep of patients with ADHD suggesting that the endogenous level of this hormone may not be sufficient to adequately set the clock in these patients[8,9].

In humans, the master clock controlling circadian rhythms is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. Within the SCN, clock genes oscillate in response to the light information transferred from the retina via the retinohypothalamic tract (RHT). This information (or zeit-geber) is then transferred to other hypothalamic nuclei and the pineal gland to modulate body temperature and production of hormones such as cortisol and melatonin. Melatonin is synthesized in the pineal gland during the dark period of the day and is involved in various physiologic functions, including sleep induction, circadian rhythm regulation, and immune response[10]. Melatonin synthesis necessitates serotonin, which is first acetylated by aryl alkylamine N-acetyltransferase (AA-NAT) and then converted to melatonin by acetyl serotonin methyl transferase (ASMT also known as HIOMT)[10]. Melatonin signaling is mainly mediated by the guanine nucleotide binding (G) protein-coupled receptors MTNR1A (MT_1) and MTNR1B (MT_2) that are expressed in the SCN but are also present in other hypothalamic nuclei, retina, immune cells,

and other peripheral organs. One of the downstream cellular effects of melatonin receptor activation is inhibition of adenylate cyclase and cAMP production[11]. GPR50 is an orphan G protein receptor with no affinity for melatonin, but as a dimer with MT_1, it inhibits melatonin signaling [12].

Twin studies previously showed that that melatonin secretion is highly heritable [13], but very little is known on the genetic and functional variability of the melatonin pathway in humans. In this study, we ascertained the genetic variability of all the genes within the melatonin pathway in a relatively large sample of 328 individuals from Sweden. These results provide evidence that mutations affecting the melatonin pathway can actually be detected in humans suggesting a higher risk for circadian rhythm dysfunction in these individuals.

Material and methods

Subjects

Individuals with ADHD (n=108, 62 males and 46 females), diagnosed according to DSM-IV criteria, were from independent families and recruited at Sahlgren University Hospital in Göteborg. The control samples (n=220, 142 males and 78 females) were all from Sweden and of European descent. The local research ethics boards reviewed and approved the study. Informed consent was obtained from probands, parents and controls.

Genetic screening of the melatonin pathway

Blood samples were collected and DNA was extracted by the phenol/chloroform method. Mutation screening was performed by direct sequencing of the PCR products. All PCRs were performed with Qiagen HotStar Taq kit. Two PCR protocols were used: (i) Standard protocol: 95°C for 15 min, followed by 35 cycles at 99°C for 30 sec, 55 to 65°C for 20 sec, 72°C for 1 to 1,5 min, with a final cycle at 72°C for 10 min; and (ii) Touchdown protocol: 95°C for 15

min followed by 20 cycles at 99°C for 30 sec, 60-50°C for 30 sec, and 72°C for 1 min, followed by 20 cycles at 99°C for 30 sec, 50° for 10 sec, and 72°C for 1 minute, with a final cycle at 72°C for 10 min. For primers and PCR conditions, see supplementary table 1. PCR products were sequenced with the BigDye Terminator Cycle Sequencing Kit (V3.1, Applied Biosystems). Samples were then subjected to electrophoresis, using an ABI PRISM genetic analyzer (Applied Biosystems). Absence of genotyping error was controlled by sequencing the PCR product with the reverse primer in a subset of patients.

The copy number variations (CNVs) within or at proximity to *AA-NAT*, *ASMT*, *MTNR1A*, *MTNR1B* and *GPR50* were ascertained using the genotyping data from the Illumina array and the PennCNV algorithm[14]. The visualization of the CNVs was performed with the genome brownser SnipPeep (http://snippeep.sourceforge.net). The *ASMT* CNV was ascertained by both Multiplex Ligation-dependent Probe Amplification (MLPA) and microsatellite gentotyping. MLPA probes were purchased from MRC-Holland (Amsterdam, Netherlands) and used according to the manufacturer protocols. Raw traces were imported into Genemapper (Applied biosystems). Deletions and duplications were detected with the Coffalyser software (MRC-Holland). The genotyping of the microsatellite located 23 bp upstream exon 4 of *ASMT* was performed by PCR reaction using the following conditions: 5'primer: (HEX)CTGGGCTACAGAGCTGAAATG, 3'primer: CTCCTGGGTTGTGCCATTTG; PCR conditions: 95°C 15 minutes; 28 cycles: 30 seconds 95°C, 30 seconds 56°C, 30 seconds 72°C; final elongation 20 minutes 72°C. PCR products were subjected to capillary electrophoresis and genotyping was performed with Genemapper (Applied biosystems).

For SNP genotyping and association analyses,

For association analysis, the genotype of all 108 cases and 96 controls (48 males and 48 females) was assessed using the Illumina 1M BeadChip in the Centre National de Genotypage

(CNG) in Paris. The HumanHap1M chip contains 1199187 markers and is estimated to tag approximately 95 % of common genomic variation in populations of European ancestry at $r^2 > 0.8$. All patients had a genotyping for more than 95% of the SNPs. SNPs with a call rate of less than 95%, as well as SNPs with genotype distributions deviating strongly from Hardy-Weinberg equilibrium in controls ($p<1x10^{-7}$), were not included in the final analysis. PLINK version 1.05 was used to perform the association analyses[15]. Using the identity by state (IBS), the inflation factor lambda was estimated to be 1.0835, suggesting that population substructure, if present, should not have any appreciable effect on the results. Odds ratios (OR) and 95% confidence intervals (CI) were estimated from logistic regression analysis. Recombination hot spots upstream and downstream of *AA-NAT*, *ASMT*, *MTNR1A*, *MTNR1B* and *GPR50* were downloaded from the HapMap homepage (http://www.hapmap.org). P values based on two-tailed tests are presented.

Functional screening of the melatonin pathway

Human cDNA for *AA-NAT* and *ASMT* were cloned in a PcDNA-dest47 vector. Flag-MT_1 and Myc-MT_2 were cloned in pcDNA3 expression vectors as previously described [16]. Mutagenesis was performed with the QuickChange II XL site directed mutagenesis kit (Invitrogen). using. Each clone was purified with Endofree Plasmid Maxi kit (Qiagen) and entirely sequenced to rule out additional mutations in the cDNA.

HEK 293 and COS cells were grown as previously described[16]. Transient transfections were performed using JetPEI (Polyplus Transfection,France) according to manufacturer's instructions. ASMTcDNAs were transfected in COS cells by electroporation, with luciferase as a reporter gene for transfection efficiency. Cells were harvested after 48h by scrapping in PBS with 4% Chaps detergent. ASMT activity was measured by radioenzymology in 50 µg of protein from cell lysates, using NAS (50 µM) and 3H-SAM (300 µM, 50 Ci/mol) as

substrates, followed by melatonin extraction with chloroform. Results were normalized with luciferase activity.

The immunofluorescence experiments and immunoblots of the MT1 and MT2 variants were performed using COSM1 cells transfected with each plasmid and seeded the day after onto sterile 25 mm polyL-lysine-coated coverslips. After 24h, cells were fixed with PBS-PFA 4% for 15 min. After a 10-min permeabilization step in PBS-Triton X-100 0.1%, cells were blocked for 1 h with 3% BSA in PBS. Cells are immunolabeled for 1 h incubation with primary antibodies: monoclonal anti-Flag at 2µg/ml (Sigma, MO) or monoclonal anti-myc 0.2µg/ml (Santa Cruz, CA), followed by 20 minutes with the secondary antibody FITC anti-mouse. For immunoblots, crude membrane preparation were performed as described previously [17]. Proteins were loaded on 10% SDS-PAGE and transferred to nitrocellulose membranes (Whatman). After blocking with 5% non fat dried milk, membranes were incubated with primary antibodies: rabbit anti-MT_1, rabbit anti-MT_2, mouse anti-p-ERK1/2 (Santa Cruz). Immunoreactivity was revealed using IRDye infrared secondary antibodies using the LI-COR Odyssey infrared imaging system (Courtaboeuf, France).

The cellular localisation of melatonin receptors was assessed on COS cells transfected with the different Flag-tagged MT_1 mutants or Myc-tagged MT_2 mutants fixed with 4% paraformaldehyde in PBS on ice for 20 min, in the presence or not of 0.1% Triton X-100. After several PBS washes, cells were incubated in PBS containing 2% BSA for 1h then in the same buffer containing anti-Flag M2 or anti-Myc 9E10 antibodies (1h on ice). Cells were then incubated with Cyanin5-coupled secondary antibody. The fluorescence was measured by FACS.

Binding experiments were performed on crude membrane preparation and 2(^{125}I)-iodomelatonin (^{125}I-MLT) (PerkinElmer) radioligand as described previously[17]. Competition

binding assays were carried out on crude membranes at 400 pM ^{125}I-MLT and increasing concentrations of melatonin. (Sigma, St Louis, MO) as described previously[18]

Calcium-Gi activation was measured on COS cells co-transfected with the mutant receptors and the chimeric Gq/i9 able to couple to the receptor as a Galphai but to activate the Galphaq-dependent pathway. Transfected cells were loaded with fluorophore using the "FLIPR calcium assay kit" (Molecular Devices, Sunnivale, Ca) and were stimulated with 10 µM melatonin. The fluorescence induced by calcium increase was recorded on a Flexstation (Molecular Devices, Sunnivale, Ca). The cAMP-Gi activation was determined by measuring cyclic AMP levels by HTRF using the "cAMP femto2" kit (Cisbio, Bagnols-sur-Cèze, France). Cells in suspension were stimulated by 5µM forskolin, alone or in the presence of 10 nM melatonin for 30 min. Samples were analyzed with a Pherastar apparatus (BMG Labtech, Offenburg, Germany). For the MAP-kinase activation, cells were stimulated with 10 nM melatonin and the kinetics of ERK1/2 phosphorylation was determined by immunoblotting. Phosphorylated ERK1/2 were detected by anti-phospho-ERK antibody (sc-7383, Santa-Cruz). Levels of loaded proteins were compared by detection of ERK2 (sc-154, Santa-Cruz).

Results

Identification of non-synonymous variants within the melatonin pathway

In order to study in depth the genetic variability of the melatonin pathway, we sequenced the coding and regulatory regions of the five genes required for melatonin synthesis (*AA-NAT* and *ASMT*) and signaling (*MTNR1A* and *MTNR1B, GPR50*). We could detect mutations modifying the protein sequence in all genes (Table 1). When considering the whole pathway, rare variations - observed in <5% in controls - were not enriched in patients with ADHD (10.6%; 23 rare variations / 216 chromosomes) compared to controls (22.6%; 50/440). For

more frequent variations, the frequency of each SNP was not significantly different in ADHD compared to controls (Table 1). Interestingly, one splice site mutation of *ASMT* and one stop mutation in *MTNR1A* were predicted to cause severe functional alterations and were detected in two independent patients with ADHD, but not in our geographically matched control sample.

The splice site mutation of *ASMT* (IVS5+2T>C) was detected in a patient with ADHD presenting with autistic traits, sleep disorder and electroencephalographic abnormalities. The mutation was inherited from a mother with attention/impulsivity problems and motor control dysfunctions. In this family, the sister of the ADHD patient was diagnosed with anorexia nervosa and also carried the splice site mutation. The *MTNR1A* stop mutation Y170X was detected in an adult female presenting with ADHD and OCD as well as social impairment and social phobia. The mutation is transmitted from the father with apparently no history of neuropsychiatric conditions. The ADHD patient has a son diagnosed with Asperger syndrome, but who doesn't carry the *MTNR1A* Y170X mutation.

Functional analysis of the rare variants within the melatonin pathway

In order to test for the functional consequences of the mutations identified in the patient with ADHD, we first transfected the control *vs* the mutant *ASMT* cDNA in COS cells and measured the enzyme activity using radioenzymatic assay. The splice site mutation (IVS5+2T>C) and the L326F identified in the patients with ADHD strongly affects ASMT activity (Fig. 2). These results obtained *in vitro* are consistent with our previous report of an *in vivo* ASMT deficiency in lymphocytes and B lymphoblastoid of patients with autism spectrum disorders (ASD) carrying the same mutations[19]. Interestingly, the *ASMT* mutations observed in the controls (D238G and P271L) were also shown to strongly alter ASMT activity (<10% of the wild type activity).

MT_1 and MT_2 receptor mutants were characterized in transiently transfected HEK 293 and COS cells. All mutants showed normal electrophoretic mobilities except for the mutant receptor carrying the Y170X stop mutation, which migrated at a lower apparent molecular weight (approximately 30 kDa) as expected (Fig. 3). Immunofluorescence microscopy experiments on intact cells and FACS analysis confirmed that MT_1-Y170X does not reach the cell surface (Fig. 4). The MT_1-G166E receptor had a tendency towards higher expression levels and was localized mainly in intracellular membrane compartments (Fig. 4). FACS analysis revealed a 60% reduction of MT_1-G166E at the cell surface indicating that this mutant is not completely devoid of surface expression.

Ligand binding properties were evaluated in ^{125}I-MLT saturation and competition bindings experiments (Table 2). All mutants did bind ^{125}I-MLT and melatonin with high affinity with the exception of the MT_1-Y170X stop mutant, which was devoid of any binding activity. MT_1-K334N, MT_2-R231H and MT_2-K243R showed significant increased K_d values for ^{125}I-MLT. As these mutations are located in the intracellular carboxyl terminal receptor tails, a direct role on ligand binding can be excluded. However, a diminished interaction with intracellular proteins participating in the stabilization of the G protein-dependent high-affinity ligand binding state of the receptor can be postulated as shown recently for the cytosolic protein Mupp1[20].

Coupling of mutant receptors to the G_i protein was assessed expressing a $G_{ai/q}$ chimera that couples G_i-coupled receptors to the phospholipase C/Ca^{2+} pathway (Fig. 5A). All mutants were active with the exception of the MT_1-Y170X stop mutant. Similar results were obtained when Gi activity was monitored by measuring melatonin promoted inhibition of the adenlyly cyclclase pathway. Functional properties of melatonin receptor mutants were further studied by measuring melatonin-promoted ERK1/2 activation. Wild type and mutant receptors showed the expected transient increase in ERK1/2 phosphorylation with comparable maximal

activation levels (Fig. 5B). The MT_1-Y170X stop mutant was inactive in this assay (not shown).

Analysis of CNVs and SNP of the melatonin pathway in ADHD and controls

In order to explore the association between CNVs and SNPs within genes of the melatonin pathway, we used the genotyping data of the 108 patient with ADHD and 96 Swedish controls generated by the 1M beadchip Illumina array. Using the PennCNV algorithm, as well as an eye detection using the viewer SnipPeep, we ascertained the presence of copy number variants (CNV) within or at proximity (<500 kb) of *AA-NAT*, *ASMT*, *MTNR1A*, *MTNR1B* and *GPR50*. Using this approach, we could not detect CNV within or at proximity of the five studied genes. The 1M beadchip Illumina array is a very robust approach for detecting large genomic imbalances (>100 kb), but deletions/duplications altering the hybridization of only a small number of probes (<5) could be missed. Indeed, we could not detect a known CNV of the *ASMT* gene that duplicates a genomic fragment from exon 1B to Exon 7[21]. We therefore used MLPA and microsatellite genotyping to ascertain the presence of this CNV in 4.9 % of the patients with ADHD and 2.2% of the controls.

For association analyses, we selected all SNPs between the two major recombination hot spots upstream and downstream of *AA-NAT*, *ASMT*, *MTNR1A*, *MTNR1B* and *GPR50*. Using this approach, 156 SNP with an average spacing of 1 SNP / 3.4 kb were tested. Several SNPs displayed a difference in allelic frequency between patients and controls (Figure 1 and Table 2). One SNP rs10830961 located within *MTNR1B* remains significant after correction for multiple testing (allelic *P*= 0.0002, genotype *P*= 0.0007). This SNP is part of a cluster of SNPs showing nominal P value for association with ADHD (rs12222793 P=0.004; rs3847554 P = 0.01; rs1387153 P= 0.01; rs2166706, P= 0.004 and rs10830961 P= 0.0002). Two SNPs in *ASMT* (rs28613595 and rs5949132) and three SNPs (rs546588, rs669228, rs694251 and

rs1899996) in *AA-NAT* provided significant nominal P values (Table 2).

Discussion

In this study, we sequenced all the genes of the melatonin pathway in 328 individuals and could detect mutations in all genes at a similar frequency in patients with ADHD and controls, suggesting that an alteration of this pathway is not restricted to patients with ADHD. Interestingly, the probands with *ASMT* mutations share several clinical characteristics including autistic traits, sleep disorder and electroencephalographic abnormalities. The *ASMT* gene was recently associated with susceptibility to autism spectrum disorders (ASD) and is located on the pseudoautosomal region 1 (PAR1) of the X and Y chromosomes, a region deleted in several individuals with ADHD and/or ASD [22-24]. The splice site mutation (IVS5+2T>C) was previously observed in five patients with ASD and in only one control from Italy[19,25]. The partial duplication of *ASMT* previously reported in 6% of patient with ASD and in 2% of controls [21]. In this study, there is a trend for an increase of this CNV is ADHD (4.9 %) compared to control (2.2%), but a higher sample size is required to detect significant association. Taken together, these results suggest that a subgroup of patients with association of ASD, ADHD and sleep problems[26] may be at higher risk for carrying mutations in the melatonin pathway. In the two families with functional *ASMT* mutations, we observed the co-occurrence of a sister with anorexia. This association may be anecdotal, but this could also suggest shared underlying genetic alterations between these two conditions as previously suggested[27].

The stop mutation (Y170X) in *MTNR1A* was only observed in the patient sample and represents the first mutation affecting melatonin receptor in human. Interestingly, this proband presents with social impairment and has a son with Asperger syndrome, but who do not carry the *MTNR1A* stop mutation. The ADHD proband carrying this non sense mutation

was also diagnosed with OCD like another proband who carry the *AANAT* A163V variation (Table 5). These two patients are the only two of our cohort with comorbid OCD.

Genotyping of 156 SNPs within the melatonin pathway, we could detect several SNPs with significant difference in the allelic frequency in ADHD compared to control. Interestingly, the signal at rs10830961 (P= 0.0004) within *MTNR1B* resists to multiple testing and is located with the same LD block of 62 Kb associated with fasting plasma glucose levels and diabetes[28-30]. The alleles of the SNPs associated with ADHD are in high LD (>0.8) with the alleles of rs1387153 and rs10830963 associated with low fasting plasma glucose and low *MTNR1B* mRNA level[28-30]. Considering these previous association studies, our results suggest that individuals with ADHD could have higher risk for lower fasting plasma glucose and lower *MTNR1B* mRNA level than the control population. To our knowledge, fasting glucose was not investigated in ADHD. However, studies using positron-emission tomography showed that global cerebral glucose metabolism was lower in patients with hyperactivity than in the normal controls[31,32]. As most energy for brain activity is derived from blood glucose, it was proposed that hypofunctionality of astrocyte glucose and glycogen metabolism could result in decreased and disorganized neuronal function in ADHD [33].

Taken together, these genetic and functional results indicate that mutations altering the melatonin pathway can be detected in humans. In our relatively small sample, mutations seem to be not specifically enriched in patients with ADHD. Nevertheless, in a subgroup of patients, loss of function mutations may alter the setting of the clock and as a consequence could increase the risk of ADHD. These genetic and functional results shed light on one new compelling candidate pathway for susceptibility to circadian rhythms alterations that could help clinicians for providing better treatments of patients with ADHD and sleep problems.

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Acknowledgements

We thank the patients and their families for participating in this study. We thank Dr. MA Ayoub and the Pharmacology facility of the Institut de Génomique Fonctionnelle (Montpellier, France) for performing calcium signaling assays. This work was supported by the Pasteur Institute, INSERM, CNRS, FP6 ENI-NET, Fondation FondaMentale and Fondation Recherche Médicale ("Equipe FRM", RJ).

Figure legends

Figure 1: Manhattan plots for 156 single-SNP tests of association for genes of the melatonin pathway in ADHD. SNPs were chosen within two hotspot of recombination flanking *AA-NAT*, *ASMT*, *MTNR1A*, *MTNR1B* and *GPR50* and tested for association in 108 patients with ADHD and 96 controls. The dotted line indicates the P value using the Bonferroni correction (P= 0,00032).

Figure 2. ASMT activity using COS cells transfected with either the wild-type (WT) or mutant ASMT. The G219X mutations removes exon 6 to 9 from the ASMT sequence and therefore mimics the effect of the IVS5+T>C splicing mutation observed in the ADHD patient. Data are means ± S.E.M. of three independent experiments each performed in duplicate. The WT ASMT activity is 8 ± 1 nmol/h/mg prot.

Figure 3. Detection of MT_1 and MT_2 mutants by SDS-PAGE. Lysates from HEK 293 cells transiently expressing the indicated receptors were separated by SDS-PAGE and analysis performed by Western blot using anti-Flag (MT_1) (**A**) or anti-Myc antibodies (MT_2) **(B)**. Similar results were obtained in three additional experiments.

Figure 4. Sub-cellular localization of MT_1 and MT_2 mutants. COS cells transiently expression the indicated receptors were permeabilized or not with triton X-100 and total and surface exposed receptors detected by immunofluorescence microcopy with anti-Flag (MT_1) (**A**) or anti-Myc antibodies (MT_2) **(B)**. Similar results were obtained in three additional experiments.

Figure 5. Signaling properties of MT_1 and MT_2 mutants. (A) HEK 293 cells transiently expressing the indicated receptors were stimulated (black bars) or not (white bars) with melatonin (1 μM) for 15 min and G_i activity determined as described in Materials and Methods. **(B)** ERK activation was measured by incubating HEK293 cells with 10 nM melatonin for the indicated times. Maximal activation, expressed as % of basal, was calculated. Data are means ± S.E.M. of three independent experiments each performed in duplicate.

Table 1. Allelic frequencies and functional consequences of *AA-NAT, ASMT, MTNR1A, MTNR1B* and *GPR50* variants identified in 108 patients with ADHD and 220 controls.

Variations	Allelic frequency (%)		Functional test
	ADHD (n=108)	Controls (n=220)	
AA-NAT			
V62I	0.46	0.23	not done
A163V	0.46	0.68	not done
G177D	0	0.23	not done
ASMT			
IVS5+2T>C	0.46	0	Defect in enzyme activity < 1% control activity
D238G	0	0.23	Defect in enzyme activity < 5% control activity
P271L	0	0.23	Defect in enzyme activity < 10% control activity
L326F	0.46	0.45	Defect in enzyme activity < 5% control activity
ASMT CNV	2.47	1.12	not done
MTNR1A			
Y170X	0.46	0	No surface expression and no melatonin binding
G166E	0.92	1.81	No surface expression, but activity as control
A266V	1.85	2.50	Trafficking and activity as control
K334N	0.92	0.45	Trafficking and activity as control
MTNR1B			
G24E	9.2	7.0	Trafficking and activity as control
R138C	0	0.45	Defect in trafficking and melatonin binding
R231H	0.46	1.13	Trafficking and activity as control
K243R	1.8	2.27	Trafficking and activity as control
GPR50			
S493R			not done
Male	0.14	0.16	
Female	0.16	0.09	
Del 502-505			not done
Male	0.48	0.45	
Female	0.56	0.45	
T532A			not done
Male	0.42	0.44	
Female	0.41	0.35	
I606V			not done
Male	0.61[a]	0.45	
Female	0.65	0.66	

[a] $P<0.05$

Table 2. Results of the association study for genes of the melatonin pathway in 108 individuals with ADHD and 96 controls.

GENE	SNP	Location	Alleles	Genotype distribution		Allelic frequency		Allelic P value	OR
			(Maj/Min)	ADHD	Controls	ADHD	Controls		
AANAT	rs546588	71943480	A/G	0/3/98	0/10/86	0.015	0.052	0.039	0.27 (0.07-1.01)
AANAT	rs669228	71949207	T/C	0/3/98	0/10/86	0.015	0.052	0.039	0.27 (0.07-1.01)
AANAT	rs694251	71950534	A/G	0/3/97	0/10/86	0.015	0.052	0.040	0.27 (0.07-1.02)
AANAT	rs1899996	71951556	G/T	0/1/100	0/6/90	0.0049	0.031	0.048	0.15 (0.02-1.3)
ASMT	rs28613595	1778157	G/A	0/18/82	3/26/67	0.09	0.17	0.023	0.49 (0.27-0.9)
ASMT	rs5949132	1856432	G/A	7/47/43	19/43/34	0.31	0.42	0.029	0.63 (0.41-0.95)
MTNR1B	rs12222793	92306695	G/A	11/55/35	28/45/23	0.38	0.53	0.0039	0.55 (0.37-0.83)
MTNR1B	rs3847554	92308474	T/C	7/48/45	21/41/34	0.31	0.43	0.012	0.59 (0.39-0.89)
MTNR1B	rs1387153	92313476	T/C	4/34/63	12/37/47	0.21	0.32	0.013	0.56 (0.36-0.89)
MTNR1B	rs2166706	92331180	C/T	6/45/50	19/43/34	0.28	0.42	0.0037	0.54 (0.35-0.82)
MTNR1B*	**rs10830961**	**92334405**	**G/A**	**5/44/50**	**20/45/29**	**0.27**	**0.45**	**0.00024**	**0.45 (0.3-0.7)**

***** In bold the result significant after correction for multiple test (156 SNPs tested; P<0.0003)

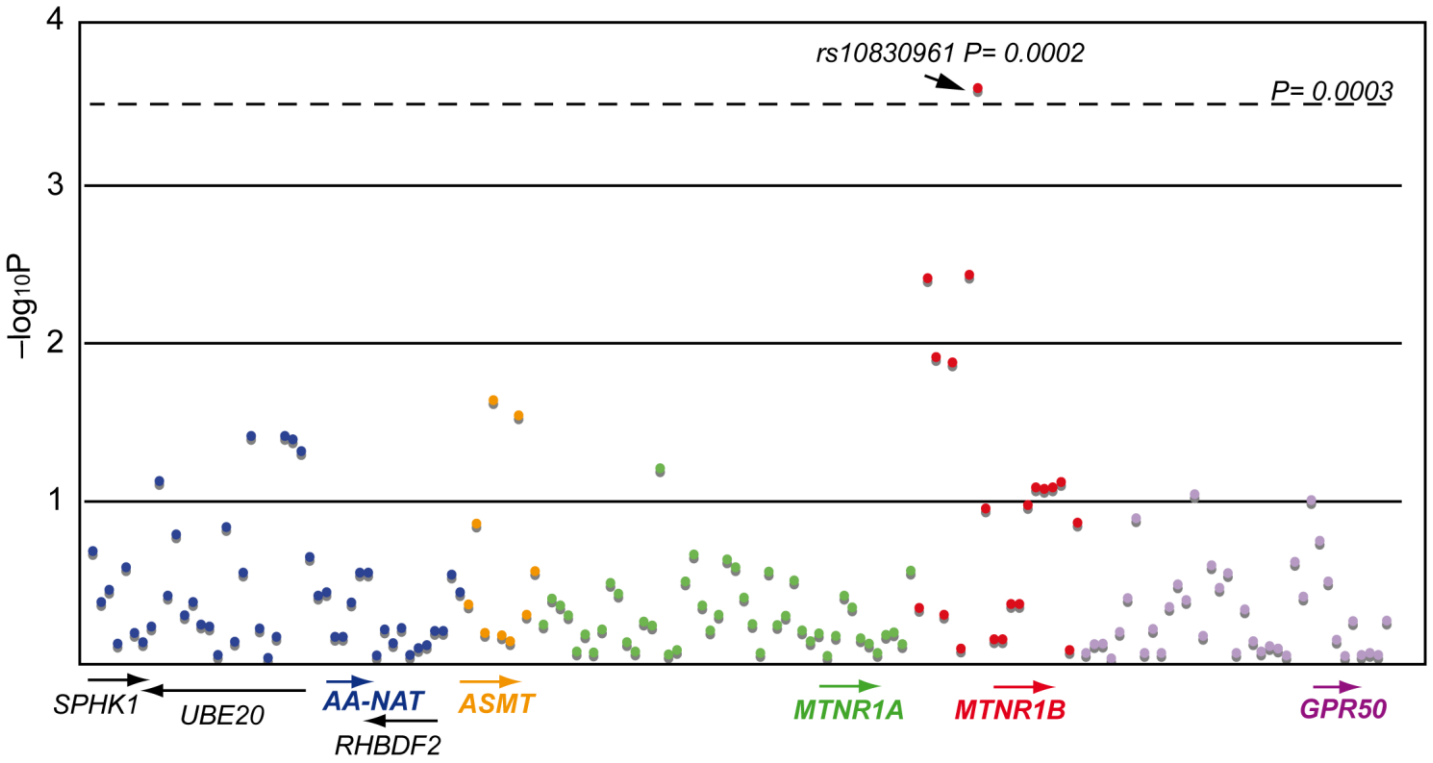
Table 3. Pharmacological characterization of MT_1 and MT_2 receptor mutants.

Receptor	K_d (pM)	K_i (pM)
MT_1		
WT	154 +/- 21	614 +/- 250
Y170X	-	-
G166E	220 +/- 20	800 +/- 150
A266V	224 +/- 43	1350 +/- 430
K334N	395 +/- 68	3270 +/- 1700
MT_2		
WT	126 +/- 20	6830 +/- 2450
G24E	268 +/- 48	3670 +/- 1600
R231H	417 +/- 59	414 +/- 290
K243R	659 +/- 29	520 +/- 270

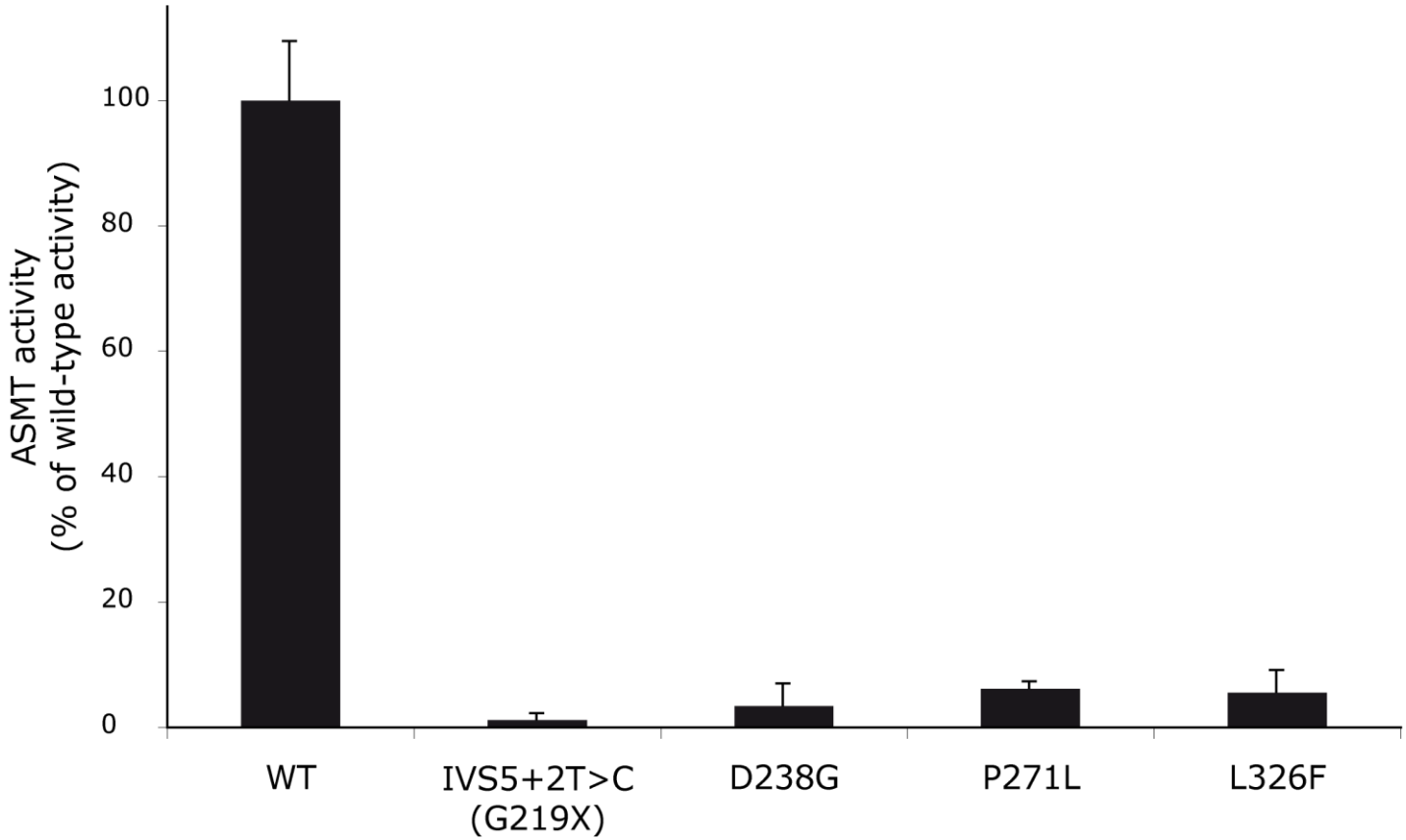
K_d values for ^{125}I-MLT were determined in radioligand saturation experiments with increasing concentration of ^{125}I-MLT. IC_{50} values for melatonin were determine in competition bindings experiments with 200 pM of ^{125}I-MLT. K_i values were calculated from IC_{50} values using the Chen-Prussof formula: $K_i = IC_{50}/1+L+k_d$. Experiments were repeated 3-7 times.

Table 5. Clinical observations of patients with ADHD carrying rare genetic variants of the melatonin pathway

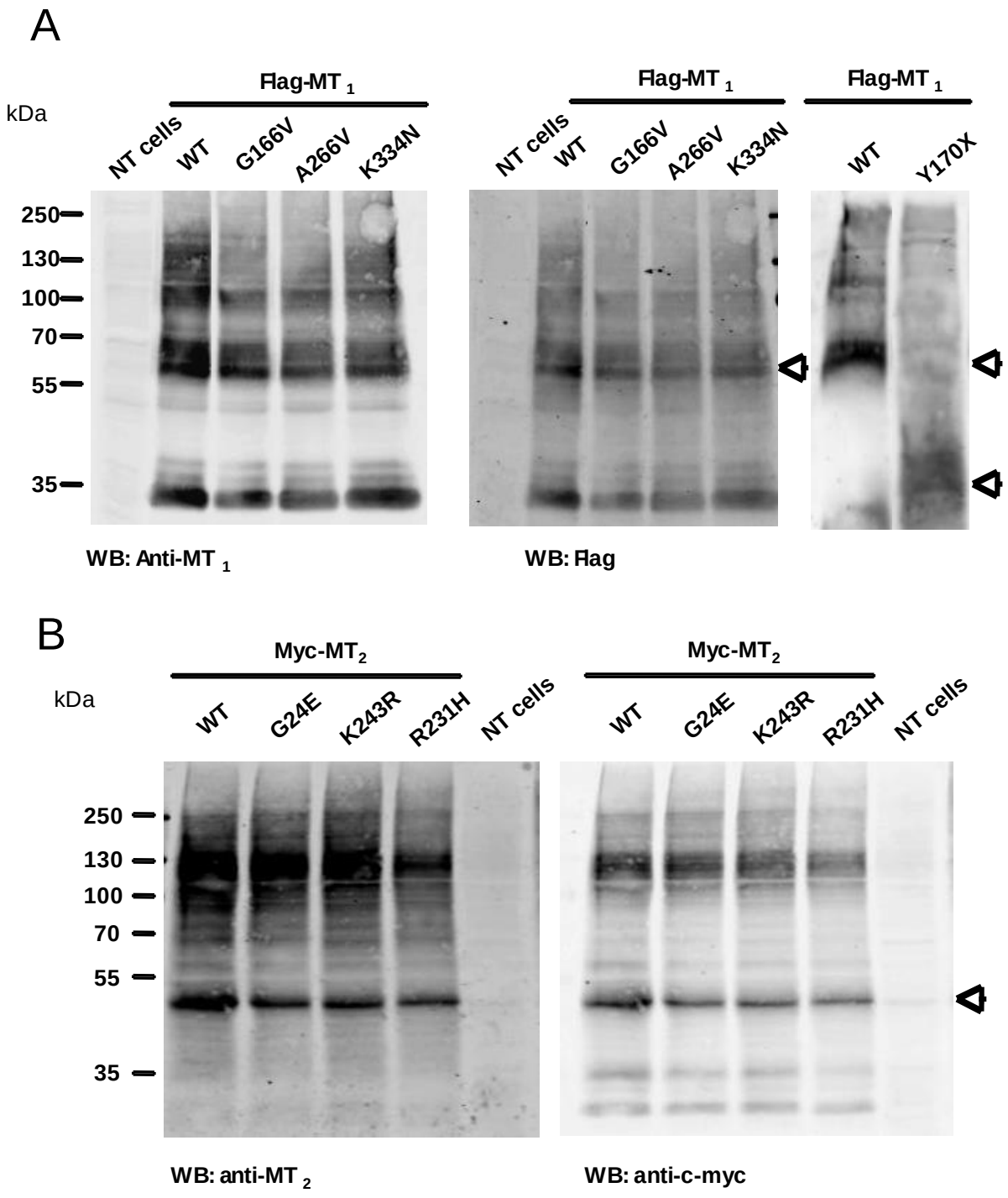
Family	Variations	Clinical observations
AD-SWE-009-JON-003	***AA-NAT*** V62I	
AD-SWE-032-LIN-003	***AA-NAT*** A163V	
AD-SWE-001-SOD-003	***ASMT*** IVS5+2T>C	
AD-SWE-064-ORN-003	***ASMT*** L326F	
AD-SWE-035-BRO-003	***ASMT*** CNV	
AD-SWE-036-EKM-003	***ASMT*** CNV	
AD-SWE-056-DUF-003	***ASMT*** CNV	
AD-SWE-079-DAH-003	***ASMT*** CNV	
AD-SWE-052-SAN-003	***MTNR1A*** Y170X	
AD-SWE-048-GRE-003	***MTNR1A*** G166E	
AD-SWE-056-DUF-003	***MTNR1A*** G166E	
AD-SWE-026-BOR-003	***MTNR1A*** A266V; ***MTNR1A*** K334N	
AD-SWE-083-WAG-003	***MTNR1A*** A266V	
AD-SWE-089-JOH-003	***MTNR1A*** A266V	
AD-SWE-096-BLO-003	***MTNR1A*** A266V	
AD-SWE-084-WIN-003	***MTNR1A*** K334N	
AD-SWE-092-BER-003	***MTNR1B*** R231H	
AD-SWE-027-LAP-003	***MTNR1B*** K243R	
AD-SWE-028-ENG-003	***MTNR1B*** K243R	
AD-SWE-054-ERI-003	***MTNR1B*** K243R	
AD-SWE-077-NES-003	***MTNR1B*** K243R	



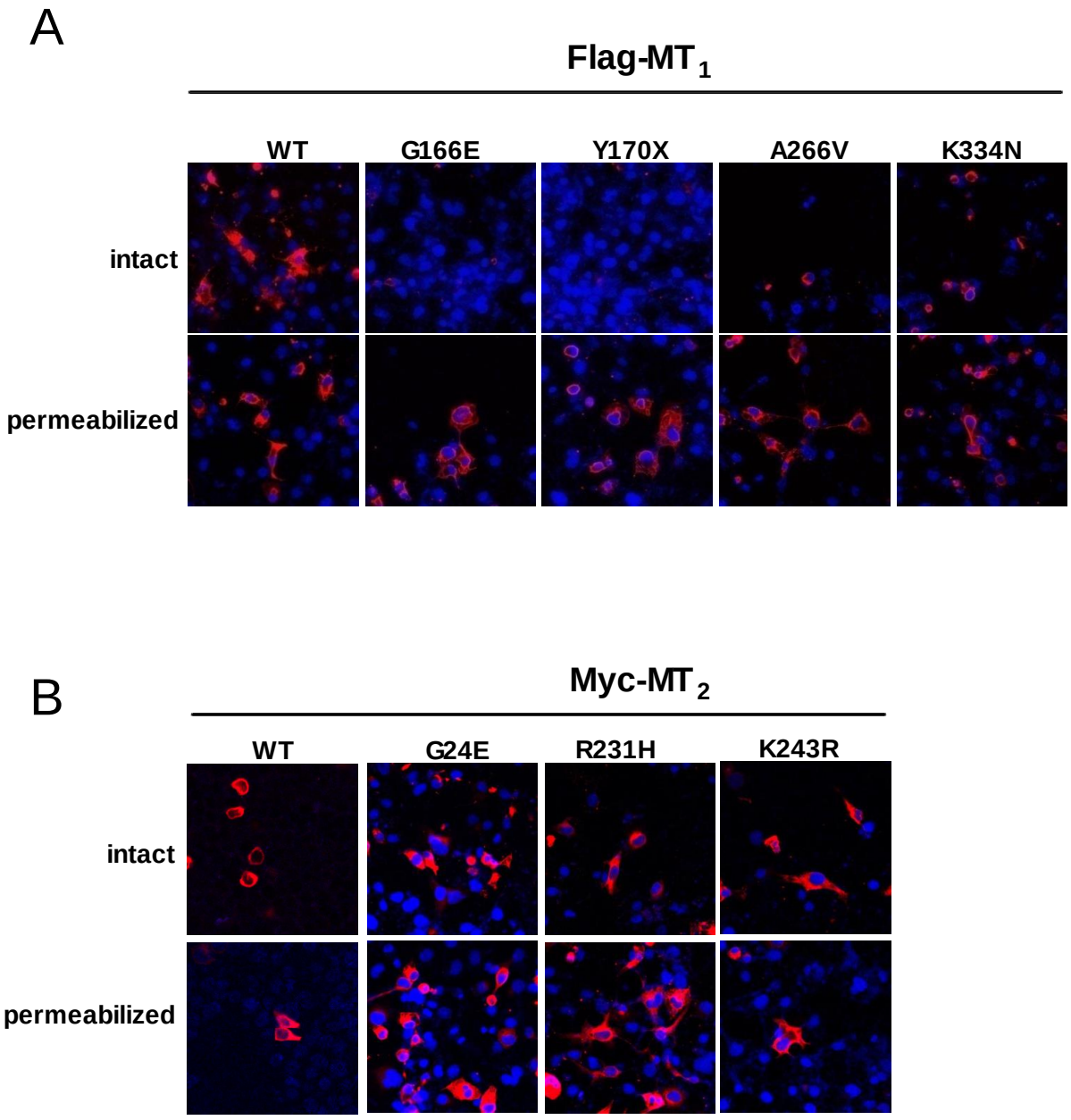
Chaste *et al.* Figure 1



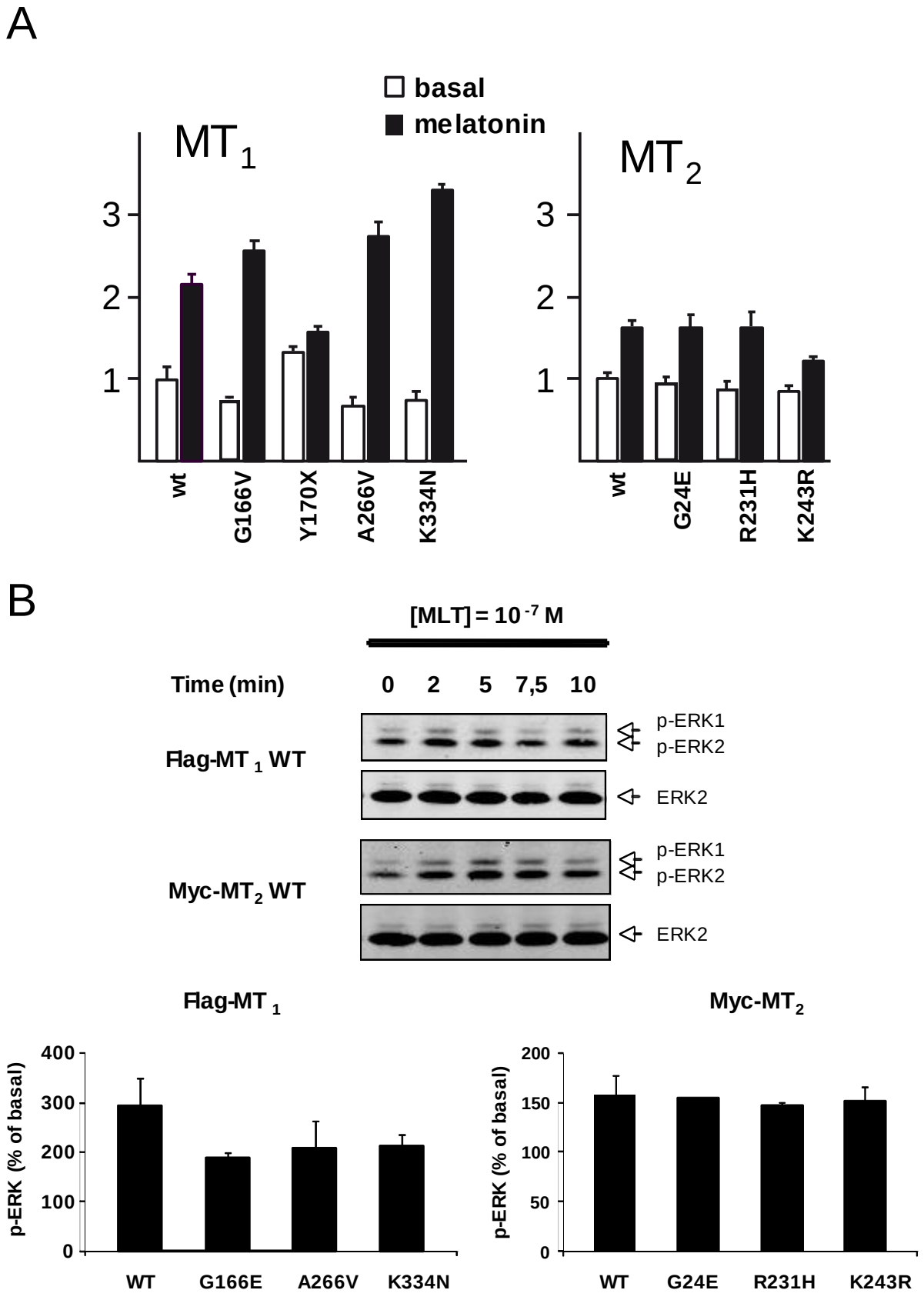
Chaste *et al.* Figure 2



Chaste *et al.* Figure 3



Chaste *et al.* Figure 4.



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