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ORIGINAL ARTICLE

Reduced platelet monoamine oxidase type B activity and lymphocyte muscarinic receptor binding in unmedicated children with attention deficit hyperactivity disorder

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Abstract

Several lines of evidence support the role of monoaminergic and cholinergic dysregulation in attention deficit hyperactivity disorder (ADHD) and the concept that peripheral blood neurotransmission indices may represent valuable surrogate CNS markers. We determined platelet MAO-B activity (p-MAO-B) and lymphocyte muscarinic cholinergic receptor binding (l-MR) in 44 unmedicated ADHD children (aged 9.1 ± 2.87 years) and in 26 age-matched controls for comparison. Lower levels of p-MAO-B (~35%) and l-MR (~55%) in ADHD were observed compared with controls. Differences were gender-dependent: p-MAO-B was reduced in males only (5.20 ± 2.99 vs 8.46 ± 5.1 nmol mg^{-1} protein h^{-1} in ADHD and controls, respectively) and l-MR in females only (ADHD vs control: 6.63 ± 1.75 and 15.30 ± 8.35 fmol 10^{-6} cells). The clinical significance was corroborated by the correlation between these markers and severity of specific symptoms: lower p-MAO-B associated with increased inattention scores (Conners' teacher-rating scale); lower l-MR associated with increased score for oppositional-defiant disorder (ODD) (SNAP-IV); and trend towards correlation between increased inattention (SNAP-IV) and lower l-MR.

Keywords: *ADHD; MAO-B; cholinergic system; blood neurochemical marker; central nervous system*

Introduction

Attention deficit hyperactivity disorder (ADHD) is a behavioural disorder found in 3–7% of school-age children (American Psychiatric Association 2000, Swanson et al. 1998). This disorder is characterized by persistent levels of hyperactivity, impulsiveness, distractibility, restlessness and inattention that is maladaptive and inconsistent with the child's developmental level. Symptoms first appear before age 7 years and may persist into adulthood. Males are affected three times more than females (Anderson et al. 1987, Baumgartel et al. 1995). Dysregulation of noradrenaline, dopamine and serotonin has been documented in ADHD (Weizman et al. 1990, Faraone et al. 2005, Malmberg et al. 2008). Children with ADHD may benefit from drugs with a noradrenergic–dopaminergic agonistic activity including psychostimulants and antidepressants (Weizman et al. 1990, Weiss & Weiss 2002). Recent studies also highlighted the possibility that the core symptoms of ADHD can result from an imbalance between the neuromodulatory effects of acetylcholine (ACh) and

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monoamines (Vakalopoulus 2007). Amelioration of ADHD symptoms has been reported in adult subjects treated with cholinergic drugs using ACh partial agonists (Wilens et al. 2006) and in youths treated with acetylcholinesterase (AChE) inhibitors (e.g. donepezil) (Wilens et al. 2000, Doyle et al. 2006) although, in a recent study, donepezil was devoid of any symptom improvement of tics in patients with comorbid ADHD (Cubo et al. 2008). Brain ACh plays an important role in memory, and attention and motivation processes that are dysregulated in ADHD. Recently, atomoxetine, a non-stimulant noradrenaline reuptake inhibitor, approved for the treatment of ADHD, has been shown to increase cholinergic neurotransmission and to ameliorate performance in laboratory animals examined by neurobehavioural tests (Tzavara et al. 2006).

The diagnosis of ADHD is based on formal structured and semistructured interviews using either US diagnostic criteria for the disorder, as defined by the Diagnostic and Statistical Manual of the American Psychiatric Association (4th edition; DSM-IV), or the European diagnostic criteria for hyperkinetic disorder (HKD), as defined by the International Classification of Diseases (10th edition; ICD-10). Both classifications are applied to children displaying developmentally inappropriate levels of inattention, hyperactivity and impulsivity that begin in childhood and cause impairment to school performance, intellectual functioning, social skills, driving and occupational functioning (Faraone et al. 2003). HKD criteria are more restrictive than the DSM-IV diagnosis of ADHD because they need a greater degree of symptom expression. Similarities and differences in DSM-IV and ICD-10 diagnostic criteria have been discussed in a recent paper by Biederman and Faraone (2005).

Both DSM-IV and ICD-10 provide well-structured, criterion-based diagnoses for ADHD and HKD. However, these methods have several weaknesses. The diagnostic items do not have developmentally sensitive definitions to help differentiate ADHD symptoms from developmentally healthy levels of inattention, hyperactivity and impulsivity. Clinicians often receive diagnostic data from multiple informants (e.g. parent and teacher, parent and teenage child, adult with ADHD and spouse), but the DSM-IV and ICD-10 provide no guidelines to integrate this information. These limitations have led to critics of ADHD describing the diagnosis as subjective and not fully credible.

With respect to ADHD, there is a great need for laboratory test systems that can enable objective diagnosis and therapeutic monitoring. Novel molecular imaging techniques may represent a valuable strategy for investigations in humans (Faraone & Biederman 2002, Nass 2005). An additional approach could be the use of biochemical markers that are detectable and quantifiable in easily accessible tissues. In recent years, blood platelets and lymphocytes have been proposed as surrogate peripheral models to investigate the brain monoaminergic and cholinergic systems in subjects with neurological diseases or neurotoxicant exposure (Manzo et al. 1996, 2001). Relevant examples of these peripheral markers include monoamine oxidase-B activity in platelets (p-MAO-B) and cholinergic muscarinic receptors in lymphocytes (l-MR) (Kawashima & Fujii 2000, Manzo et al. 2001, Ruchkin et al. 2005).

Given the relevant impact of monoaminergic and cholinergic dysregulation in ADHD, the present study aimed to (1) measure p-MAO-B activity in blood platelets and l-MR binding in blood lymphocytes of medication-free ADHD children and adolescents compared with age-matched controls, and (2) evaluate the correlation between these peripheral neurochemical parameters and the severity of ADHD symptomatology.

Materials and methods

Subjects

All infants were enrolled from the Child Neuropsichiatry Unit of the IRCCS Fondazione Casimiro Mondino (Pavia, Italy), from March 2005 to April 2008. We recruited a total of 44 Caucasian children (39 males and 5 females) between 4 and 15 years old (mean age 9.1 ± 2.87 years), with the diagnosis of ADHD.

Twenty-six children were also enrolled (19 males and 7 females) as control subjects who were aged between 7 and 17 years (mean age 11.68 ± 3.3 years).

Clinical interview: inclusion and exclusion criteria

The diagnosis of ADHD was established according to the DSM-IV-TR criteria (American Psychiatric Association 2000) by a senior child and adolescent psychiatrist following a psychiatric interview. The children and parents were invited to an assessment using: (1) the Kiddie Schedule for Affective Disorders and Schizophrenia for School-aged Children, Present and Lifetime versions (KSADS-PL) (Kaufman et al. 1996, 1997), a semistructured interview for psychiatric disorders; (2) the Conners' Rating Scale for Parents and Teachers (Conners 1997); (3) the SNAP-IV Rating Scale (26 items) (Swanson, Nolan and Pelham questionnaire) (Swanson et al. 1983). The evaluation also included the Children's Depression Rating Scale (CDRS) to assess the presence of depression (Poznanski et al. 1984) and the Screen for Child Anxiety-Related Emotional Disorders (SCARED) (Birmaher et al. 1997).

Although learning disabilities were not tested systematically, subjects were required to be of normal intelligence (IQ ≥85, Raven matrix ≥5th to 10th percentile for age) as assessed by clinical evaluation, a Wechsler Intelligence Scale (WISC III), or a non-verbal intelligence test (Raven matrix test). Only five children presented mild or moderate mental retardation. The subjects were also tested to assess any other comorbid psychiatric disorder (e.g. anxiety and mood disorders, substance abuse, psychotic disorders, conduct and oppositional-defiant disorders (ODD)), affective disorders or any comorbid condition requiring use of medications.

Studies were conducted to exclude a thyroid dysfunction or any other serious medical illness, and routine blood analyses as well as ECG measurements were also performed.

Controls underwent a comprehensive interview (KSADS-PL) to exclude the presence of any psychiatric disorder, in particular those requiring use of psychoactive medications. All participants were drug-free for at least 2 months when enrolled for the study. Information about cigarette smoking was obtained by asking the subjects, at the time for the blood sampling, and no one declared they smoked (0% boys, 0% girls).

The study met all regulatory requirements and was conducted in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 2000. It was approved by the ethical committee of Fondazione Mondino, Pavia, Italy and informed consent was obtained from the parents and participants.

Psychometric instruments

Concomitantly with the blood samples collection, clinical evaluation and psychometric rating scales were completed for all ADHD participants, in particular:

(1) The KSADS-PL, to evaluate any psychiatric comorbid condition;
(2) The Conners' Parent and Teacher Rating Scales, a short version (diagnosis for hyperactivity and/or inattention if patients met at least six criteria each);
(3) The SNAP-IV scale conducted by the interviewer (26 items, diagnosis was considered present if patients had a score of at least 1.78 for inattention, at least 1.44 for hyperactivity and at least 1.67 for ODD);
(4) The CDRS scale, to assess the presence of depression (diagnosis from a score of at least 40);
(5) The screen for child anxiety related emotional disorders (SCARED), to assess the presence of comorbid anxiety disturb (diagnosis from a score of at least 25).

Neurochemical markers

The biochemical analyses were performed according to the Certification program for the quality assurance UNI EN ISO 9001:2000 in laboratory medicine.

Isolation and storage of blood cells

Blood samples from ADHD and control children (about 10 ml) were collected between 08.00 and 10.00 h into tubes containing EDTA. They were immediately processed to isolate lymphocytes for MR binding or platelets for MAO-B activity as previously described (Coccini et al. 2005). The lymphocytes were resuspended in the freezing solution (90% plasma obtained from autologous blood kept on ice + 10% DMSO, dimethylsulfoxide), while the platelet-rich plasma (PRP) was diluted with 10% DMSO. Immediately after, the cells were gradually frozen at –80°C for 24 h and thereafter stored in liquid nitrogen.

Chemicals

[^{3}H]-Quinuclidinyl benzilate ([^{3}H]-QNB) (49 Ci mmol^{-1}) and scintillation fluid Biofluor were obtained from NEN Life Sciences Products (Boston, MA, USA), while ^{14}C-PEA (ethyl-1-^{14}C-phenylethylamine hydrochloride, 41.8 mCi mmol^{-1}) was from Du Pont de Nemours (Florence, Italy). All the other chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Platelet MAO-B activity

On the day of the analysis, PRP was thawed and the platelets were isolated by centrifugation at 500*g* for 10 min, resuspended in Na^+/K^+ phosphate buffer (5 mM) pH 7.4 and counted by Coulter Counter (Instrumentations Laboratory). Following centrifugation at 16 000*g* for 10 min, the pellet was resuspended in the same Na^+/K^+ phosphate buffer supplemented with 5% bovine serum albumin, 10 mM dithiothreitol and 2.5 mM EDTA, homogenised for 20 s, and further diluted with buffer to a concentration of 20×10^6 platelets per 0.4 ml.

The activity of p-MAO-B was determined radiochemically in duplicate samples as described by Coccini et al. (2002) using 10 µM ^{14}C-PEA as the substrate. Specific activity was determined in the presence of 100 µM pargyline hydrochloride. The mixture contained 0.4 ml of tissue homogenate in a final assay volume of 1 ml. The reaction was started by addition of the ^{14}C-PEA and stopped by adding 0.25 ml HCl (4 N) after a 10 min-incubation at 37°C. Deaminated reaction products were toluene-extracted and the radioactivity contained in a 1 ml aliquot of the organic phase was counted by a scintillation counter. The enzyme activity was expressed as nmol mg^{-1} protein h^{-1}. The mean platelet protein concentrations in platelet homogenate were 0.187 ± 0.029 mg ml^{-1}.

Lymphocyte muscarinic receptor binding

Binding of the specific muscarinic antagonist [3H] QNB to lymphocytes was performed as described by Coccini et al. (2005). One million lymphocytes were incubated, in the presence or absence of 100 µM atropine, with 27 nM [^{3}H]QNB in Hank's buffer (0.5 ml total volume) using 96-plate wells. Following 60 min incubation at 27°C, the reaction was stopped by adding 2 ml of ice-cold phosphate-buffered saline (PBS). Samples were rapidly filtered through Unifilter GF/C 96 plate wells using a Unifilter cell harvester (Packard, Milan, Italy) and washed three times with ice-cold PBS. Then the Unifilter plate wells were air dried and counted for radioactivity in 100 µl of Microscint (Packard) in a Top Count NXT (Packard) scintillation counter. Each sample was assayed in triplicate and data were expressed as fmol 10^{-6} cells.

Internal quality control

As a measure of quality assurance, a pooled human lymphocyte or platelet preparation, taken as the internal quality control of each test, was always tested along with the unknown samples. The intra-assay variation (CV) was <10% for p-MAO-B activity and 10–20% for l-MR binding calculated on assayed $n = 3$–6 replicates of different aliquots of the same platelet or lymphocyte pool. The CV for the interassay precision ranged between 20 and 30% for both tests.

Statistical analysis

Descriptive statistics on raw values for l-MR and for p-MAO-B was performed on the whole sample and according to subgroups identified by gender. All results are expressed as mean ± SD. Two-tailed unpaired Student's *t*-test was used for between-group comparison. Pearson correlation test was used as appropriate. The data were analysed using the statistical software package SPSS (SPSS Inc., Chicago, IL, USA, 1999).

Results

Behavioural characteristics

Psychometric rating scales for all ADHD subjects are presented in Table 1. Compared with girls, boys had lower average scores in hyperactivity and inattention evaluated by the Conners' Parent Rating Scale, but higher scores in hyperactivity and inattention evaluated by the Conners' Teacher Rating Scale although only one female subject could be examined.

Platelet MAO-B activity

p-MAO-B activity was measured in all participants. p-MAO-B activity, males and females combined, was lower (36%) in the ADHD group compared with the controls (5.39 ± 3.13 vs 8.43 ± 5.11 nmol mg^{-1} protein h^{-1}, $t = 2.7$, df = 36.24, $p < 0.05$), the 95% confidence interval (CI) being 6.37–10.50 for controls and 4.44–6.34 for ADHD subjects. A statistical difference was found in males only, possibly due to the low number of female subjects examined in both ADHD and control groups ($n = 5$ in ADHD and $n = 7$ in controls) (Figure 1). The male p-MAO-B values in the ADHD and control groups were 5.20 ± 2.99 ($n = 39$) and 8.46 ± 5.1 ($n = 19$) nmol mg^{-1} protein h^{-1}, respectively ($t = 2.5$, df = 24.2, $p < 0.05$).

Lymphocyte muscarinic receptor binding

l-MR binding values measured in blood samples from ADHD and control subjects are shown in Figure 2. The combined value (males plus females) of ^{3}H-QNB binding was lower (30%) in the ADHD group compared with the controls (9.56 ± 4.97 vs 13.42 ± 9.89 fmol 10^{-6} lymphocytes, respectively, $t = 1.9$, df = 33.47, $p = 0.059$) with the 95% CI 8.01–11.12 for ADHD subjects and 8.95–17.16 for controls. The difference was apparent in females only (about 55%) with average l-MR binding values of 6.63 ± 1.75 fmol 10^{-6} cells in the ADHD

Table 1. Behavioural characteristics in attention deficit hyperactivity disorder patients. The scores (mean ± SD) for each used rating scale are presented.

		Total		Male		Female	
Scale		Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*
Conners' parent[a]	Hyperactivity	6.7 (1.4)	33	6.6 (1.4)	28	7.2 (1.3)	5
	Inattention	6.9 (1.4)	33	6.8 (1.4)	28	7.6 (1.3)	5
Conners' teacher[a]	Hyperactivity	6.3 (1.2)	19	6.5 (1.1)	18	4	1
	Inattention	6.7 (1.2)	20	6.8 (1.1)	19	5	1
SNAP- IV	Hyperactivity	2.0 (0.5)	32	2.0 (0.5)	29	2.0 (0.8)	3
	Inattention	1.9 (0.8)	31	1.9 (0.9)	28	2.0 (0.4)	3
	ODD	1.8 (0.8)	26	1.8 (0.8)	25	1.2	1
SCARED		15.7 (8.8)	18	16.5 (9.3)	15	11.7 (2.5)	3
CDRS		31.9 (8.7)	17	31.8 (7.8)	15	32 (18.3)	2

[a]A short version.The SNAP-IV scale for hyperactivity and inattention was similar in both genders, and also the overall score on the CDRS scale was similar in boys and girls. Higher scores in boys were obtained by the SCARED.

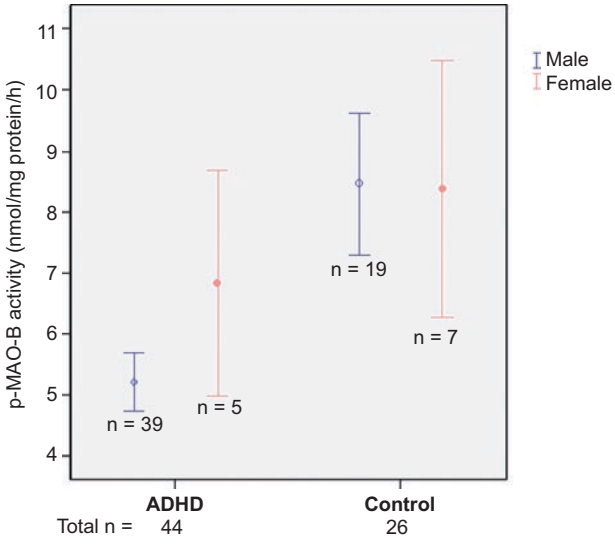

Figure 1. MAO-B activity in platelets (p-MAO-B) of subjects (girls and boys) with attention deficit hyperactivity disorder (ADHD) and controls. The results are expressed as mean ± SE.

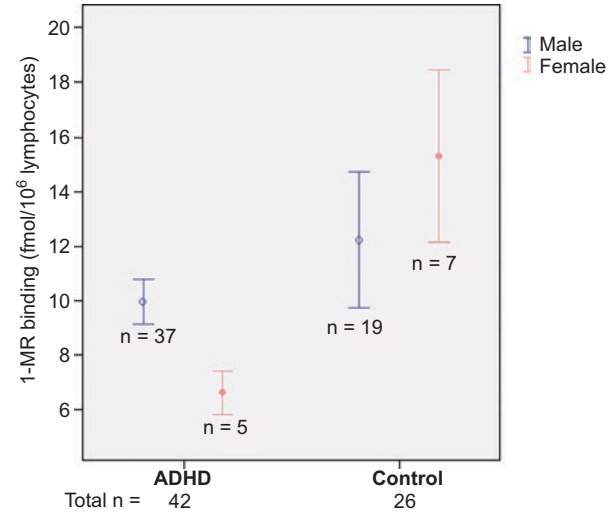

Figure 2. Muscarinic receptor binding in lymphocytes (l-MR) of subjects (girls and boys) with attention deficit hyperactivity disorder (ADHD) and controls. The results are expressed as mean ± SE.

group vs 15.30 ± 8.35 fmol 10^{-6} cells in the controls (p <0.05) (Figure 2).

Correlations

Correlations, between each neurochemical marker and the psychometric measures were evaluated considering the whole ADHD group (male plus female subjects) due to the low number of girls. Summary data are presented in Table 2.

A significant inverse correlation was found between the Inattention Conners' teacher score and p-MAO-B activity (r = -0.44, n = 20, p <0.05) (Table 2, Figure 3).

Table 2. Pearson's coefficient (r) correlations between platelet MAO-B value and psychometric measures.

	Total ADHD subjects		
Scale	*r*	*n*	*p*-Value
Conners' parent: hyperactivity	0.04	33	0.80
Conners' parent: inattention	0.03	33	0.80
Conners' teacher: hyperactivity	0.08	19	0.70
Conners' teacher: inattention	−0.44	20	0.04*
SNAP-IV: hyperactivity	−0.12	32	0.50
SNAP-IV: inattention	0.25	31	0.16
SNAP-IV: ODD	0.26	26	0.20
SCARED	−0.44	18	0.06
CDRS	−0.18	17	0.48

Tests for differences were calculated for each pair of correlations, namely p-MAO and each psychometric test.

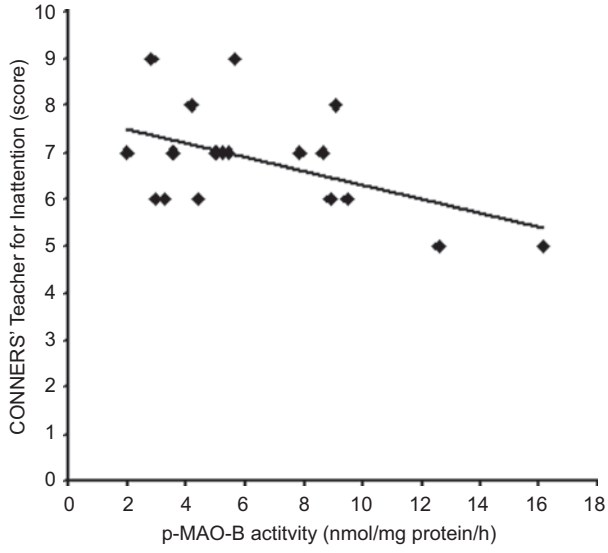

Figure 3. The scatterplot shows the distribution of the pair of variables, namely p-MAO-B activity and Conners' teacher for inattention score, using Pearson's correlation (r = -0.44, n = 20, p = 0.04). The p-MAO-B activity values (mean ± SD, n = 20) were 6.27 ± 3.62 nmol mg^{-1} protein h^{-1}.

l-MR binding inversely correlated with the SNAP-IV ODD (r = −0.51, n = 24, p <0.05) (Table 3, Figure 4) and a trend towards significance was observed between the SNAP-IV Inattention score and l-MR binding (r = −0.36, n = 29, p = 0.06).

There were also a negative association trend between p-MAO-B and SCARED scores as well as between l-MR and CDRS scores.

Discussion

The major finding of this exploratory study was the lower platelet MAO-B activity and lower lymphocytes MR binding in untreated ADHD subjects compared with normal healthy controls. The observed differences

Table 3. Pearson's coefficient (r) correlations between lymphocyte muscarinic receptor (l-MR) value and psychometric measures.

	Total ADHD subjects		
Scale	r	n	p-Value
Conners' parent: hyperactivity	–0.26	31	0.14
Conners' parent: inattention	–0.22	31	0.22
Conners' teacher: hyperactivity	0.07	19	0.78
Conners' teacher: inattention	0.24	20	0.31
SNAP-IV: hyperactivity	0.10	30	0.58
SNAP-IV: inattention	-0.36	29	0.06
SNAP-IV: ODD	–0.51	24	0.01**
SCARED	0.27	17	0.30
CDRS	–0.48	16	0.06

Tests for differences were calculated for each pair of correlations, namely muscarinic receptor binding and each psychometric test.

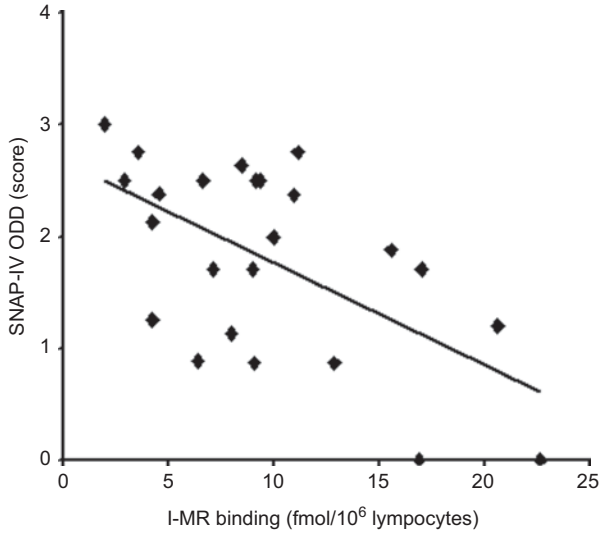

Figure 4. The scatterplot shows the distribution of the pair of variables, namely muscarinic receptor binding in lymphocytes (l-MR) and SNAP-IV ODD score, using the Pearson's correlation (r= -0.51, n=24, p=0.01). The l-MR binding values (mean ± SD, n=24) were 9.65 ± 5.53 fmol 10^{-6} lymphocytes.

between ADHD subjects and controls were gender-dependent in that lower p-MAO-B was found in males only and lower l-MR in females only.

The clinical significance of these findings was corroborated by the observations indicating an inverse correlation between either p-MAO-B activity values or l-MR binding values and the severity of specific ADHD symptoms. ADHD subjects showed lower p-MAO-B associated with increased inattention scores (Conners' Teacher Rating Scale). No significant correlation was observed between p-MAO-B and the inattention score of Conners' Parent Rating Scale.

At the same time, lower MR binding in lymphocytes was significantly associated with increased score for ODD (SNAP-IV). A trend towards correlation between increased inattention (SNAP-IV) in ADHD patients and lower l-MR binding was also observed.

There are several reports in the literature indicating changes in p-MAO-B activity in association with psychiatric and personality disorders (Danielczyk et al. 1988). In particular, numerous studies have addressed in adult subjects the association of low platelet MAO activity and schizophrenia, obsessive-compulsive disorder (OCD) and borderline personality disorder (Kaneda et al. 2001, Arrojo et al. 2007, Verkes et al. 1998), alcoholism (Anthenelli et al. 1995, Coccini et al. 2002, Snell et al. 2002), pathological gambling (Blanco et al. 1996) and bulimia (Carrasco et al. 2000). The observations in children are limited. Lower p-MAO-B activity was reported in hyperactive boys by Shekim et al. (1986). An association between low p-MAO-B activity and symptoms of ODD was also found in adolescent girls (Malmberg et al. 2008). It was suggested that abnormal levels of p-MAO-B activity may represent a surrogate indicator reflecting altered central serotonergic capacity associated with hyperactivity and attention deficit in adolescents (Kiive et al. 2007). A significant positive correlation between platelet serotonin concentration and impulsive symptoms was also recently observed in ADHD children (Hercigonja Novkovic et al. 2009). Consistent with these findings, is the notion that ADHD may be ascribed to hypoactivity of brain monoamines, namely hypofunction of dopaminergic, noradrenergic and serotonergic pathways (Weizman et al. 1990). Serotonergic processes are involved in several behavioural traits such as aggression and impulsiveness, which are frequently associated with ADHD (Halperin et al. 1997, Mitsis et al. 2000). Serotonin and dopamine exert regulatory control over each other, suggesting that serotonin, in addition to dopamine, is likely to be linked to ADHD (Oades 2008). The mechanism behind the association between p-MAO-B and aggressive behaviour is difficult to confirm. However, studies in healthy individuals (human and non-human primates) demonstrate, with appropriate methods and precaution of confounding factors, a significant correlation between cerebrospinal fluid levels of the serotonin metabolite 5-HIAA and p-MAO activity (Fahlke et al. 2002). This seems in good agreement with the finding that adult men, children and youths and non-human primates with a tendency for aggressive behaviour have low levels of 5-HIAA in the CSF as well as low MAO activity in platelets (Oreland et al. 2004). It needs to be underlined that there are two MAO subtypes, MAO-A and MAO-B that share 70% similarity in amino acid sequence. *In vitro* experiments with brain extracts have shown that MAO-A and MAO-B have different but overlapping substrate specificities. Dopamine and tyramine are good substrates for both types of MAO. Serotonin is a better substrate for MAO-A than for MAO-B. *In vivo*, the substrate specificities of the MAO subtypes seem to be regionally modulated. In human platelets, MAO is exclusively of the B-type and

has the same amino acid sequence as MAO-B in brain (Oreland 2004). Several hypotheses have been claimed to explain the association between p-MAO-B and personality/behaviour (Oreland et al. 2004). One of these hypothesizes that p-MAO is correlated to brain MAO-B, which would then contribute to the creation of the personality traits of interest as a result of an effect on the rate of monoamine neurotransmitter degradation and, further, on monoaminergic activity. Another hypothesis suggests that p-MAO may directly influence the level of some trace amine, which might be of importance for behaviour. A further hypothesis suggests that p-MAO is regulated together with other mitochondrial enzymes, and then low platelet MAO might indicate generally low mitochondrial function, which might result in lower efficiency in a particularly vulnerable transmitter system, such as the serotonin system. The most supported hypothesis considers p-MAO activity as a genetic marker, e.g. for the 'capacity' of central monoamine system and particularly of serotonergic. Such a common genetic control could occur via common gene promoter sequences and coregulation of p-MAO and monoamine transmitter genes. The pattern shown for p-MAO-B activity resembles results shown with regard to MAO-A genotype. In fact, besides p-MAO-B activity investigation, the gene encoding the MAO-A enzyme (MAO-A VNTR) is also frequently investigated to evaluate the behaviour–serotonergic capacity association (Oreland et al. 2007, Malmberg et al. 2008).

The core symptoms of ADHD have been attributed to imbalance between the neuromodulatory effects of monoamines and ACh (Vakalopoulos 2006, 2007). Based on this model, it has been proposed that impulsivity and hyperactivity may result from impaired dopaminergic inhibition and remodelling of muscarinic-mediated prepotent responses. The central cholinergic system and MR activation have long been associated with cognitive function. For instance, the cholinergic MRs are involved in several CNS functions, including learning and memory (Levine et al. 2001). Gene technology studies using MR-deficient mice further support the critical role of MRs in higher brain functions (Wess 2003). Animal studies also provide evidence for the cortical cholinergic pathways in mediating subtypes of attention other than memory per se (Leblond et al. 2002). The core symptoms of ADHD include inattention and hyperactivity/impulsivity. Dopaminergic dysfunction has long been considered central to the aetiology because of the marked response to indirect dopamine agonists methylphenidate and d-amphetamine. The study of ADHD provide a unique insight into the relative roles of the monoamines in declarative memory, that is, ability to organize and categorize prefrontal-related strategies and working memory function. Several investigations indicate that working memory dysfunction may have heuristic value in explaining the inattentive dimension so characteristic of ADHD. Premotor theory would predict a relative explicit versus implicit memory deficit associated with monoaminergic dysfunction (e.g. reduction in the monoamine levels). According to premotor theory a hypofunctioning dopaminergic system would predict a very specific neuromodulator imbalance. The theory also offers insight into the second symptom dimension of hyperactivity/impulsivity. An intriguing proposal is that the core deficits of hyperactivity and impulsiveness result from a failure of extinction of previously reinforced or learned behaviours (Sagvolden et al. 2005a). The findings are consistent with a failure of antagonism of the motivating circuits served by simple premotor networks and muscarinic cholinergic modulation as a consequence of dopaminergic hypoactivation (Vakalopoulos 2007). Consistent with the latter interpretation are the findings in the spontaneously hypertensive rat (SHR), which is one of the best animal models of ADHD (Hand et al. 2006, Pardey et al. 2009) exhibiting the typical ADHD behavioural characteristics such as hyperactivity, inattention, resistance to extinction and hypersensitivity to reinforcer delay. Indeed, altered dopamine function in the SHR conforms to the theoretical rationale for ADHD, providing construct validity for this animal model (Sagvolden et al. 2005b). Notably, SHRs also exhibit immune deficiencies as well as lower levels of ACh in lymphoid tissues and lower ChAT mRNA expression in circulating mononuclear leukocytes (Fujimoto et al. 2001, Fujii et al. 2008). Interestingly, it has been postulated that blood ACh is involved in regulating the T cell-dependent immune response through stimulation of the muscarinic and/or nicotinic receptors existing on the lymphocytes.

Based on the peculiarity that MRs are expressed in non-neural tissues such as lymphocytes, peripheral MRs have been applied clinically as predictors of pharmacological response in subjects treated with psychotropic drugs and to investigate hypotheses involving neurochemical disturbances in affective disorders and neurological diseases. For example, alteration of l-MR binding has been evidenced in Alzheimer's (Tayebati et al. 1999), Parkinson's (Rabey et al. 1991) and Menière's diseases (Masuyama et al. 1996), as well as in Gilles de la Tourette syndrome (Rabey et al. 1992). Altered expression of peripheral blood lymphocyte receptor subtypes (i.d. M3 and M4) have been suggested to be related to degrees of cognitive impairment in patients with Alzheimer's disease (Tayebati et al. 2001). In view of the easy accessibility of peripheral lymphocytes for research and diagnostic purposes, assay of lymphocyte MRs may contribute to the assessment of cholinergic dysfunction in ADHD. Among the potential surrogate cholinergic markers, lymphocytes express as well as muscarinic ACh receptor subtypes several nicotinic ACh receptor

subunits. It has been found that during immunological reactions (for example by phytohaemagglutinin activation), stimulated T cells have the ability to synthesise and release ACh, which in turn acts in an autocrine and/or paracrine fashion on both muscarinic and nicotinic receptors on T cells to modulate immune function (Fujii et al. 2008).

In summary, this study provides evidence that levels of surrogate peripheral markers of cholinergic and monoaminergic neurotransmissions are reduced in ADHD children. The results indicate a relationship between levels of p-MAO-B activity and l-MR binding, and specific ADHD symptoms such as inattention and ODD in unmedicated patients. These findings support the notion that p-MAO-B and l-MR binding can be valuable in clinical studies of ADHD and possibly for monitoring the effectiveness of pharmacological treatments.

Clearly, there are some limitations in this exploratory study to be considered. First, our experiments have focused on total MR binding while specific MR subtypes are likely implicated in CNS disorders (Wess et al. 2007). Second, genetic variables (Oreland et al. 2007, Dick et al. 2007, Li et al. 2007) that are known to affect neurotransmission parameters in neuropsychological illness (Ramoz et al. 2009) were not considered as possible response modifiers in the ADHD subjects. Moreover, the gender effect on the clinical presentation of the ADHD disorder (Biederman et al. 2002; 2005) is an open question that needs to be further investigated using a larger sample of recruited subjects including a similar number of girls and boys.

Another critical issue is the conundrum of the multiple informants (parents, teachers, youths and healthcare professionals) who do not necessarily agree on diagnosis (Nass 2005). In particular, we found the Conners' parent and teacher ratings unusually similar, whereas the correlations with p-MAO-B measures in Table 2 do show a marked difference (i.e. Conners' inattention: teachers vs parents). As the number of children rated varied considerably, the importance of investigating a larger sample size of subjects once again emerges. Furthermore, our exploratory findings call for more work designed to evaluate the subtypes of ADHD and comorbidity in order to add more insight and meaning into ADHD.

As the study included a small number of subjects, who were evaluated at a single time point, additional studies are needed to assess the predictive value of these markers in relation to pharmacotherapy or to the persistence or timing of remission of the disorder.

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