BDNF concentrations and daily fluctuations differ among ADHD children and respond differently to methylphenidate with no relationship with depressive symptomatology

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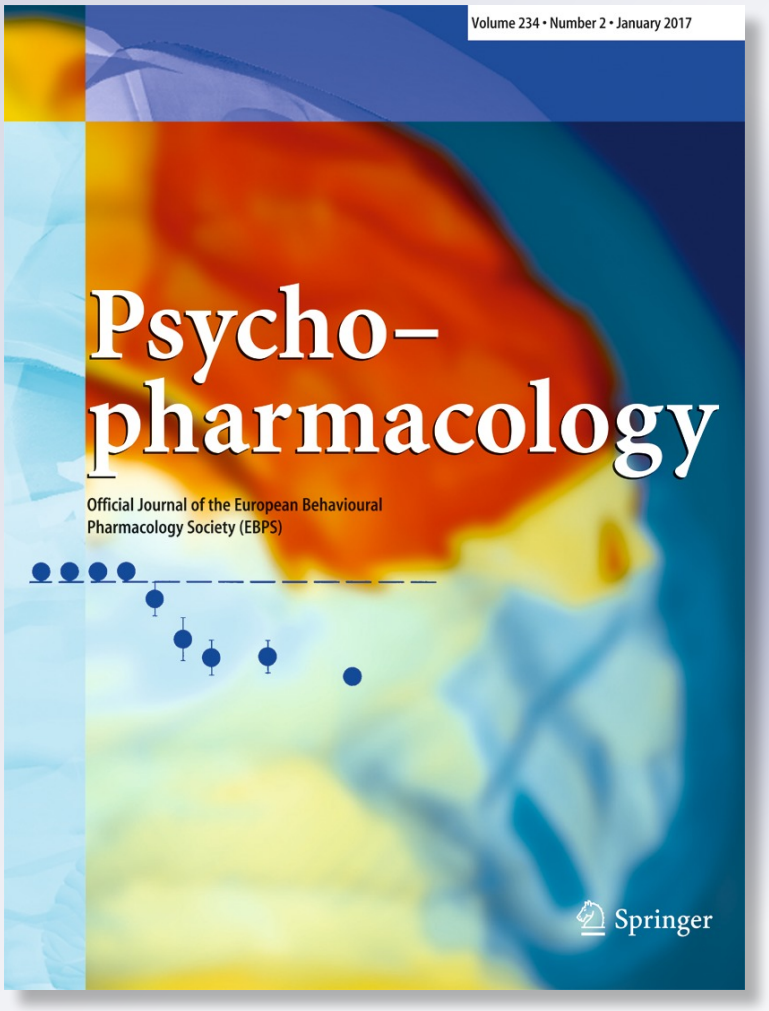

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ORIGINAL INVESTIGATION

BDNF concentrations and daily fluctuations differ among ADHD children and respond differently to methylphenidate with no relationship with depressive symptomatology

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Abstract
Rationale Brain-derived neurotrophic factor (BDNF) enhances the growth and maintenance of several monoamine neuronal systems, serves as a neurotransmitter modulator and participates in the mechanisms of neuronal plasticity. Therefore, BDNF is a good candidate for interventions in the pathogenesis and/or treatment response of attention deficit hyperactivity disorder (ADHD).
Objective We quantified the basal concentration and daily fluctuation of serum BDNF, as well as changes after methylphenidate treatment.
Method A total of 148 children, 4–5 years old, were classified into groups as follows: ADHD group (n = 107, DSM-IV-TR criteria) and a control group (CG, n = 41). Blood samples were drawn at 2000 and 0900 hours from both groups, and after 4.63 ± 2.3 months of treatment, blood was drawn only from the ADHD group for BDNF measurements. Factorial analysis was performed (Stata software, version 12.0).
Results Morning BDNF (36.36 ± 11.62 ng/ml) in the CG was very similar to that in the predominantly inattentive children (PAD), although the evening concentration in the CG was higher (CG 31.78 ± 11.92 vs PAD 26.41 ± 11.55 ng/ml). The hyperactive–impulsive group, including patients with comorbid conduct disorder (PHI/CD), had lower concentrations. Methylphenidate (MPH) did not modify the concentration or the absence of daily BDNF fluctuations in the PHI/CD children; however, MPH induced a significant decrease in BDNF in PAD and basal day/night fluctuations disappeared in this ADHD subtype. This profile was not altered by the presence of depressive symptoms.
Conclusions Our data support a reduction in BDNF in untreated ADHD due to the lower concentrations in PHI/CD children, which is similar to other psychopathologic and cognitive disorders. MPH decreased BDNF only in the PAD group, which might indicate that BDNF is not directly implicated in the methylphenidate-induced amelioration of the neuropsychological and organic immaturity of ADHD patients.

Keywords ADHD · ADHD subgroups · Human · Children · BDNF · Methylphenidate · Serum · Daily rhythms · Depressive symptoms · Comorbidities

This work is part of María-José Ruiz-Ramos doctoral thesis.

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Introduction

Theories supporting the neurobiological basis of attention deficit hyperactivity disorder (ADHD) are based on two complementary models, both of which are based on the dysregulation of interacting neural pathways, i.e. inhibitory noradrenergic fronto-cortical activity on dopaminergic striatal structures (Wiltschko et al. 2010) and increasing

dopamine circuits in the limbic system (Nigg and Casey 2005). Although ADHD has a multifactorial origin with a strong genetic component (Biederman and Faraone 2005), there are age-related changes in discrete brain areas and connectivity that parallel behavioural improvement and increased efficiency in cognitive task performance (Matthews et al. 2014).

In addition to the key features of ADHD, which include the core problems of inattention, hyperactivity and impulsivity, the vast majority of ADHD patients have at least one comorbid condition, e.g. conduct disorder, depressive symptoms or sleep disorders. While both ADHD subtypes have depressive symptoms with severities equal to non-ADHD psychiatric control groups and are greater than community control groups, externalising behaviour problems and aggression appear to be related to the hyperactive–impulsive ADHD symptom domain and overall ADHD symptom severity (Connor and Ford 2012).

Among the genetic factors related to ADHD, polymorphisms related to the activity of brain-derived neurotrophic factor (BDNF) seem to be correlated with the incidence, clinical manifestations, endophenotypes or treatment response in ADHD (Hong et al. 2011). BDNF is highly expressed in the central nervous system, and it is able to cross the blood-brain barrier in both directions. BDNF operates by binding to the tropomyosin-related kinase B (TrkB) receptor, enhancing the growth and maintenance of several monoamine neuronal systems, serving as a neurotransmitter modulator and participating in neuronal plasticity mechanisms (Simchon Tenenbaum et al. 2015), such as long-term potentiation and learning (Thoenen 1995).

The pharmacological approach to ADHD treatment includes psychostimulants, norepinephrine reuptake inhibitors and alpha-2 agonists (Molina-Carballo et al. 2016). The norepinephrine transporter (NET) is known to transport dopamine (DA) with a higher affinity than norepinephrine (NE) with preferential activation within the prefrontal cortex, where dopamine transporter (DAT) density is low and NET density is higher. Stimulants, such as methylphenidate, block the reuptake of DA and NE (Wilens 2008), increasing the catecholaminergic tone acting through alpha-2 receptors, thus activating the hypofunctional medial prefrontal and orbitofrontal cortex. BDNF is closely related to DA pathways and dopaminergic function (Li et al. 2010). DAT/NET recovery via enhanced BDNF signalling is important for the pharmacological activities of methylphenidate (MPH) (Nam et al. 2014).

Conversely, dopamine intervenes in the developmental regulation of striatal BDNF expression, suggesting that the effects of dopamine on inhibitory GABAergic cells could be intertwined with BDNF action. Therefore, BDNF plays an important role in the development and functioning of the dopamine system because the induction of BDNF expression might constitute a downstream response to D1-like dopamine receptor activation (Williams and Undieh 2009).

The serotonergic system is influenced by genetic variations in BDNF (Henningsson et al. 2009). Conversely, the administration of selective serotonin reuptake inhibitor antidepressants enhances BDNF gene expression. BDNF promotes the survival and differentiation of serotonin neurons (Lyons et al. 1999). Serotonin and BDNF are two distinct signalling systems that play regulatory roles in many neuronal functions, including survival, neurogenesis and synaptic plasticity. Additionally, they both regulate the development and plasticity of neural circuits involved in mood disorders, such as depression and anxiety (Martinowich and Lu 2008). Additionally, the circadian regulation of cognition might involve rhythms of BDNF/TrkB expression in the hippocampus (Martin-Fairey and Nunez 2014).

Therefore, BDNF is crucial for dopaminergic, serotonergic and glutamatergic neurotransmission. In addition to BDNF regulation of the monoaminergic systems, it plays a pivotal role in synaptic remodelling during development (Garcia et al. 2010; Zhang et al. 2013).

In addition to strong genetic factors for ADHD occurrence, social (non-intact family, young maternal age, low paternal education and low income) and environmental (premature birth/low birth weight, prenatal smoking or illicit drug use, maternal depression and paternal history of antisocial behaviour) risk factors also affect ADHD, especially if these factors are present in the pre and early postnatal periods during the development of the brain (Sagiv et al. 2013). In particular, all of these factors are associated with reduced neonatal weight, which is a recognised biomarker for future risk of morbidity. Both newborns with low birth weights and those who are large for their gestational ages have increased rates of metabolic and mental disorders, including ADHD, anxiety and depression. Abnormal BDNF gene activity probably underlies the mechanism by which these early life adverse experiences persistently modify brain and behavioural plasticity (Roth and Sweatt 2011). These mechanisms are related to DNA methylation, leading to disruptions in the cell cycle during development and gene expression in adulthood. Alterations in BDNF with a placental origin during gestational growth might be one mechanism by which DNA methylation is altered in the developing CNS (Lipsky 2013).

Based on these experimental and clinical data, which were recently reviewed (Liu et al. 2015) and postulated a role for neurotrophic BDNF in both the pathogenesis and the response to the treatment of ADHD, the aims of our study were to examine the BDNF serum levels and their daily fluctuations in children with ADHD before and after chronic methylphenidate treatment and to investigate whether these relationships were related to depressive symptomatology.

Methods

Subjects

A total of 148 children (115 males and 33 females) between the ages of 5 and 14 years old (mean 9.61 ± 2.54 years) were included in a prospective, quasi-experimental, open clinical study in a hospital-based sample, which primarily reported objective neuroendocrine measurements of response.

The sample consisted of the following two groups: an ADHD group, in which each included patient was assessed at least twice and, consequently, could be considered as his/her own control, and a control group, which served only as a reference. A total of 107 children who met the DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders, fourth edition, text revision)/ICD-9 (International Classification of Diseases, ninth revision) criteria for ADHD were included in the ADHD group after completing the clinical protocol to exclude main comorbidities. We included a control group (CG, $n = 41$), mainly composed of the siblings ($n = 35$) of the ADHD subjects (recruited simultaneously to his/her brother or sister) or unrelated subjects ($n = 6$) who were healthy children, all of whom had adequate academic performance.

Clinical methods

Each child with ADHD was assessed at least twice. We obtained a personal medical history and physical examination and distributed the following documents: (a) DSM-IV-TR criteria assessment, which was completed by the child's teacher; (b) an EDAH scale (Spanish acronym for the evaluation of deficit of attention and hyperactivity scale) (Sánchez et al. 2010), in duplicate, one was completed by the teacher and the other by the child's parents; (c) the Children's Depression Inventory (CDI), which was completed by subjects aged ≥8 years old; and (d) a sleep diary, which was completed for 1 week. The EDAH contains some of the main criteria recommended in the DSM-IV-TR to aid in identifying children with ADHD and conduct disorder (CD). The EDAH questionnaire is a 20-item scale (Farré-Riba and Narbona 1997) that utilises structured observation by teachers and is divided into two 10-item subscales for ADHD and CD. Based on EDAH scores, the ADHD group was quantitatively subclassified into the following two clinical subgroups: children with predominant attention deficits (predominantly inattentive children (PAD); if attention deficit (AD) >9, hyperactivity-impulsivity (HI) <10 and total score <30) and children with the predominant hyperactive–impulsive subtype with comorbid conduct disorder (PHI/CD; if AD <10, hyperactivity (H) >9 and/or total score >29). Therefore, of the 78 children who were included in the PHI/CD group, 34 (44 %) met the criteria for the diagnosis of HI without CD. Of the 44 children with symptoms of CD, 33 showed a predominance of symptoms of HI among the symptoms of CD, while the remainder of the children (11/78; 14 %) had a prevalence of symptoms of CD among the symptoms of HI. Only 26 of 78 children in this group (33 %) did not meet further criteria for attention deficit.

The d2 Test is a measure of attention, particularly visual attention. The d2 Test measures processing speed, rule compliance and quality of performance, allowing for a neuropsychological estimation of individual attention and concentration performance by the quantification of the following two scoring keys: errors of omission and errors of commission. The test has been fully validated and includes extensive norms, according to age, sex and education.

The CDI is a self-report assessment of depression for children, the two subscales of which (negative mood and negative self-esteem) consist of items that are most unique to depression and least related to anxiety. To define the subgroups, we considered the sum of both subscales with a quantitative cut-off of >17 points considered pathological. The depressive symptoms were assessed through interviews with the parents at baseline and during the clinical follow-up and were quantified by the CDI completed by each child.

All of the children were evaluated with an abbreviated intelligence test as a screening of cognitive ability (Kaufman Brief Intelligence Test (KBIT)), and they also completed the Spanish version of the Sleep Diary of the National Sleep Foundation for 1 week (data not shown), with the ADHD group completing the diary once again after treatment.

Written informed consent was obtained from all of the parents and children ≥12 years of age, and informed assent was obtained from all of the participants. No control subjects were treated with any drug for ethical reasons, and only one assessment was performed. The study design and outcome variables were approved by the Hospital Ethics Committee and the Health Research Fund of the Spanish Ministry of Science and Innovation.

The exclusion criteria were as follows: (1) KBIT <85, (2) preexisting or actual treatment for epilepsy, (3) other treatments for ADHD or other conditions and (4) revocation of previous informed consent.

The somatometric characteristics, vital signs and haematological and biochemical data from the study groups are provided in Table 1

Treatment

The only drug used in the study was prolonged release methylphenidate (PRMPH, OROS formulation), initially at 0.5 mg/kg/day. The dosage was adjusted as a function of the response and tolerance to treatment (absence of adverse symptomatology). The mean initial dose of methylphenidate was 25.81 ± 10.35 mg, and the final dose at the time of the second evaluation was 31.85 ± 10.68 mg. At inclusion, all of the

Table 1 Somatometrics, vital signs and haematological and biochemical data for the control group and the ADHD groups

	Control (n = 41)	PAD (n = 29)	PHI/CD (n = 78)	Statistics	
				t	p
Age (year)	10.22 ± 2.58	9.59 ± 2.77	9.33 ± 2.41	1.81	0.07
Sex (M/F)	30/11	24/5	61/17	X^2 = 0.67	0.41
Height (m)	1.47 ± 0.18	1.37 ± 0.19	1.37 ± 0.16	3.06	0.001**
Weight (kg)	44.179 ± 15.14	35.54 ± 13,12	36.87 ± 16.21	2.39	0.003**
BMI (kg/m^2)	19.8 ± 4.14	18.40 ± 3.53	18.90 ± 4.40	1.14	0.254
HR (bpm)	79.81 ± 12.96	77.75 ± 11.49	79.22 ± 10.36	0.37	0.71
SBP (mm Hg)	105.42 ± 13.80	102.58 ± 14.50	101.36 ± 13.97	1.41	0.16
DBP (mm Hg)	64.11 ± 8.57	65.73 ± 17.51	63.57 ± 10.95	0.241	0.81
Hb (g/l)	13.87 ± 1.02	13.79 ± 0.84	13.90 ± 0.79	0.40	0.69
Hct (%)	39.18 ± 2.40	37.84 ± 6.87	40.19 ± 5.83	0.39	0.70
MCV (fl)	78.16 ± 8.90	77.49 ± 7.51	79.66 ± 8.99	0.37	0.71
Iron (mg %)	84.68 ± 29.76	77.73 ± 29.02	89.12 ± 31.31	0.10	0.92
Ferritin (ng/l)	38.12 ± 13.53	40.32 ± 15.55	41.94 ± 21.25	1.07	0.29
TSH (μIU/l)	2.44 ± 1.26	2.62 ± 0.98	3.00 ± 1.44	1.77	0.08
KBIT score	107.88 ± 12.29	103.16 ± 10.35	104.06 ± 11.15	0.77	0.21

M male, *F* female, *BMI* body mass index, *HR* heart rate, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *t t* test for unrelated samples, *MCV* mean corpuscular volume, *TSH* thyroid-stimulating hormone, *KBIT* combined punctuation of the Kaufman abbreviated intelligence test

*Significant differences between the CG and the ADHD group with p values (0.001 and 0.003) already included in the column. None of the comparisons of the data for the ADHD groups were significant. Data are expressed as the mean ± SD

patients were naive to any medication, and no other treatment (pharmacological or psychological) was administered before the conclusion of the protocol.

Measurements

None of the samples were obtained in the presence of an acute or severe illness. Blood samples were obtained at 20:00 and at 09:00 the day following inclusion. In the ADHD group, after 4.61 ± 2.29 months of daily methylphenidate administered early in the morning, the identical study protocol was repeated. Serum was separated into 0.5-ml aliquots for freezing at −30 °C until analysis.

Analytical methods

BDNF was measured using the enzyme-linked immunosorbent assay (ELISA) kits (IBL International, Hamburg, ref. RB59041) with a minimum sensitivity of 80 pg/ml and coefficients of variability of 10 (intra-assay) and 12 % (inter-assay).

Statistics

To achieve the objectives of the study, factorial analyses were conducted as described below. For comparisons between the EDAH and CDI scores (ordinal variables), Wilcoxon signed-rank tests (paired samples) were used for inferential statistics. For comparisons between patients (cases) and each variable in the study, the factors in the factorial models were as follows: (a) groups with two categories: PAD and PHI/CD groups; (b) patients, nested in groups and subgroups (CDI); (c) hour, with two categories, day and night, crossed with the groups; and (d) time, with two levels, before and after treatment; this factor was a crossed factor with group and hour. Group, hour and time were fixed effect factors; and patients were a random effect factor. Comparisons between cases and controls were performed because there was only one measurement for the controls using the same analysis repeated in two different situations, which were as follows: baseline in cases compared with controls and after treatment in cases compared with controls. The factorial model had the following three factors: (1) group with three categories (controls, PAD and PHI/CD), (2) subjects (controls and patients) nested in groups and patients nested in CDI subgroups and 3) hour, with two categories, day and night, crossed with group. Group and hour were fixed effect factors, and subjects were a random effect factor. For both types of comparisons, an ANOVA table was built, and higher interactions were determined. If these interactions were significant, multiple pairwise comparisons were performed using Bonferroni correction, and, if not, these corrections were applied to the principal effects in the table. The experimental quantities for these comparisons were not '*t*', as

expected, because we used '*z*', the normal approximations for *t*s, because of the global sample sizes. The analyses reported were crude analyses, and adjusted analyses by age and sex were performed using ANCOVA methodology. In all of the cases, the interactions were studied for levels less than 0.15, and the latest comparisons were considered significant at $p < 0.05$ after applying the correction. When analysing the variances in different groups, homogeneous transformations were performed on data using natural logarithms to achieve uniformity. We used the Stata statistical package, version 12.0, for all of the analyses.

Results

The mean heights and weights were significantly higher in the control group in part due to the slightly higher mean age than the ADHD group; however, there were no differences in BMI (Table 1). After treatment, the average height of the patients was unchanged, while the weight decreased, which was expected and previously reported (Molina-Carballo et al. 2013).

Table 2 shows the clinical course data (EDAH, d2 and CDI scores) for the ADHD group separated into diagnostic groups. Although not significantly different (data not shown), the incidences of depressive symptoms were 20.7 and 24.4 % in the PAD and PHI/CD groups, respectively and were more common in girls than boys (34.8 vs 20.2 %, respectively). More than 80 % experienced improvements (EDAH scores) after methylphenidate treatment based on the parental evaluation data.

BDNF comparisons between groups

At baseline, the children with ADHD (30.16 ± 12.63 ng/ml) had significantly ($z = 2.19$, $p = 0.028$) lower concentrations of BDNF than the control children (34.39 ± 11.88 ng/ml); these differences persisted after adjusting for age and gender (Fig. 1). The values were higher in the morning (33.19 ± 12.43) with high statistical significance ($z = 2.76$, $p = 0.006$) compared with the night values (29.16 ± 12.41).

In the comparison of the CG with the two ADHD subgroups, the mean value of BDNF levels of the control group showed no differences with the PAD-ADHD (30.86 ± 12.9 ng/ml), but there were differences ($z = 2.26$, $p = 0.024$) between the CG and the PHI-CD group (29.91 ± 12.57 ng/ml). Considering only the hour-of-day factor, there were significant differences between day and night ($z = 2.79$, $p = 0.0052$) in the whole sample, with higher values in the morning. These differences were erased in the post hoc pairwise comparisons, after including both subgroups and hour of day as factors.

Accounting for the presence or absence of depressive symptomatology showed no differences in the morning or at night in either of the ADHD groups (Table 3). There was a trend ($z = 1.87$, $p = 0.06$) towards lower morning values in the PHI/

Table 2 Means and standard deviations for the EDAH scale, d2 and CDI scores for the control group and the ADHD subgroups at the study inclusion

Test	Score	Control group	ADHD groups							
			PAD group				PHI/CD group			
			Baseline	Post-PRMPH	Statistics[a]		Baseline	Post-PRMPH	Statistics[a]	
		Mean ± SD	Mean ± SD	Mean ± SD	*z*	Sig	Mean ± SD	Mean ± SD	*z*	Sig
EDAH scale	AD	3.75 ± 3.13	11.07 ± 1.49	8.67 ± 3.01	−2.672	0.008	10.33 ± 2.94	8.72 ± 2.9	−4.244	<0.001
	H	4.08 ± 2.73	5.59 ± 2.51	5.83 ± 2.55	−0.287	NS	10.44 ± 2.73	8.18 ± 2.95	−4.852	<0.001
	CD	5.11 ± 3.26	6.89 ± 3.18	7.39 ± 5.81	0.687	NS	15.81 ± 4.93	12.26 ± 5.08	−4.217	<0.001
	AD + H	7.83 ± 4.70	16.76 ± 2.24	14.5 ± 4.18	−1.699	NS	20.75 ± 4.42	16.84 ± 5.19	−5.035	<0.001
	Global	12.94 ± 6.50	23.56 ± 4.46	21.89 ± 8.75	−1.166	NS	36.56 ± 7.74	29.14 ± 9.46	−4.889	<0.001
d2—attention test	O	15.39 ± 17.98	17.57 ± 23.33	22.50 ± 42.99	−0.314	NS	16.09 ± 23.59	8.94 ± 12.38	−1.609	NS
	C	9.43 ± 13.94	15.96 ± 18.84	11.89 ± 27.47	−1.891	0.059	15.06 ± 22.95	10.28 ± 17.17	−2.566	0.01
	CON	123 ± 41.56	83.61 ± 34.92	111.1 ± 47.48	2.586	0.01	88.59 ± 39.79	117.21 ± 52.4	4.195	<0.001
	Total	323.11 ± 82.58	242.8 ± 79.74	303.7 ± 84.87	2.949	0.003	247.68 ± 77.22	300.96 ± 93.8	4.328	<0.001
CDI	NM	2.38 ± 2.28	4.56 ± 4.29	4.11 ± 3.23	−0.666	NS	5.54 ± 3.68	5.91 ± 6.07	−2.591	0.01
	NSE	4.47 ± 3.32	7.89 ± 4.08	7.00 ± 3.25	−1.720	NS	7.99 ± 3.26	7.12 ± 3.16	−3.463	0.001
	Total	6.85 ± 5.06	12.44 ± 7.30	11.11 ± 6.12	−1.930	0.054	13.44 ± 6.24	12.33 ± 7.80	−3.476	0.001

EDAH Spanish acronym for the evaluation of deficit of attention and hyperactivity scale, *AD* attention deficit, *H* hyperactivity, *CD* conduct disorder, *O* number of omissions, *C* number of commissions, *CON* concentration score, *NM* value of the 'negative mood' subscale of the Childhood Depression Inventory (CDI), *NSE* value of the 'negative self-esteem' subscale of the CDI, *z z* value on Wilcoxon signed-rank test

[a] Statistical comparison of the ADHD groups between baseline and post-PRMPH data

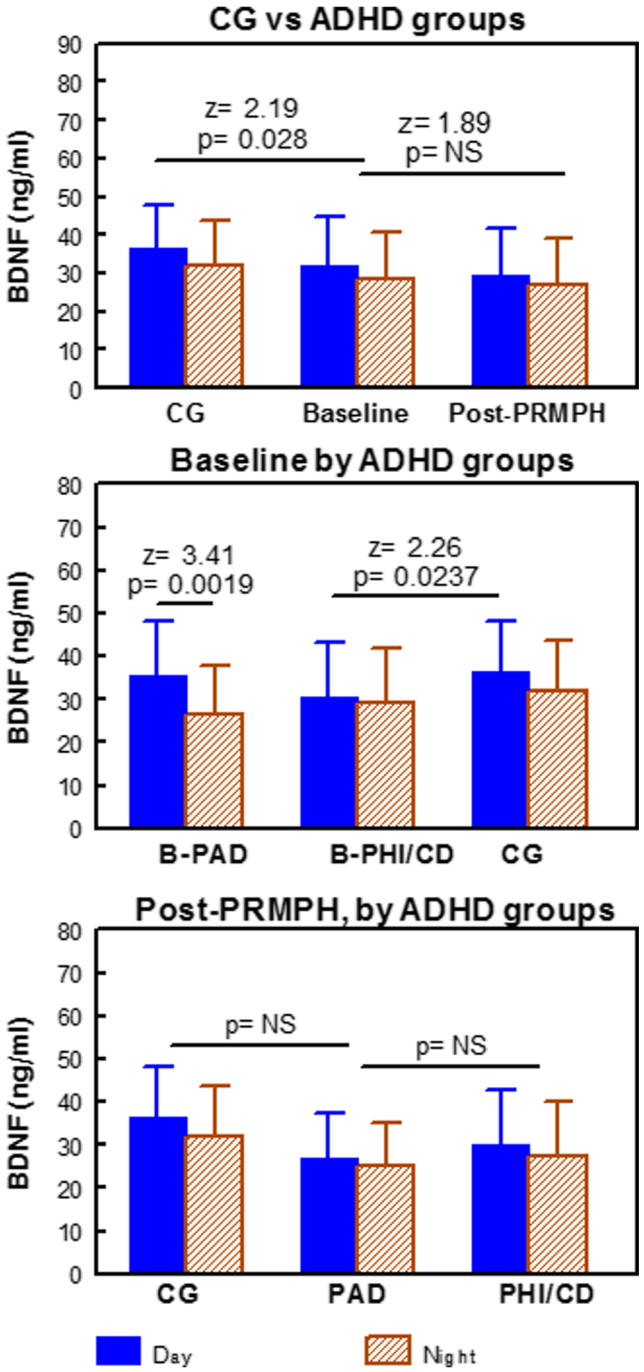

Fig. 1 Comparisons of the BDNF serum levels among groups, subgroups and subtypes. The control group and the ADHD subgroups (*top figure*), the ADHD groups and the control groups at baseline (*middle figure*) and the control group and the ADHD subtypes post-chronic treatment with PRMPH. Additionally, the *middle figure* includes the significant difference between the day and night for the baseline PAD group. *PAD* predominantly attention deficit group, *PHI/CD* predominantly hyperactive–impulsive/conduct disorder, *B* baseline, *Post* post-prolonged release methylphenidate (PRMPH)

CD group in the absence vs the presence of comorbid depressive symptoms (28.83 ± 11.47 vs 35.64 ± 15.2 ng/ml). After accounting for depressive symptoms in the factor analysis, the day/night differences continue to have high statistical significance ($z = 2.79$, $p = 0.005$) and higher morning concentrations.

BDNF response in the ADHD group

After treatment, the BDNF levels showed a non-significant decrease (28.05 ± 12.20 vs 30.16 ± 12.63 ng/ml) with similar day/night differences ($z = 3.03$, $p = 0.0025$) from baseline.

In the PAD group, the baseline BDNF concentrations were very similar to those in the CG, with greater day/night differences at baseline that reached significant differences (Fig. 2). Methylphenidate induced a significant decrease in the BDNF values to the lowest BDNF values (26.97 ± 10.3 ng/ml, $z = 1.83$, $p = 0.021$ in the morning; 25.05 ± 10.21 ng/ml, p = NS at night) as shown in Fig. 2. In contrast, in the PHI/CD group, methylphenidate did not induce any changes in the BDNF values in the morning or at night. Including depressive symptomatology as a factor did not change the results. A significant difference ($z = 2.24$, $p = 0.025$) in the day values before and after treatment was only observed in the PAD group (Table 3).

The analysis by ADHD groups only showed a trend towards BDNF differences between the basal morning values in the absence of depressive symptoms ($z = 1.95$, $p = 0.051$), with a higher concentration in the baseline PAD group than post-treatment.

Discussion

Our study is the first to report BDNF concentrations and daily fluctuations in children diagnosed with ADHD grouped according to ADHD groups (PAD and PHI, including patients with comorbid conduct disorders) and subgroups (comorbid depressive symptoms) both before and after PRMPH treatment.

The data reported here supported previous reports on child (Molina-Carballo et al. 2012) and adult patients (Corominas-Roso et al. 2013), which showed decreased serum BDNF in ADHD patients compared with healthy controls. Additionally, we reported that prolonged treatment with methylphenidate induced further decreases in serum BDNF due to decreases in predominantly inattentive children without any changes in predominantly hyperactive children and with no influence on depressive symptomatology. Although the PHI/CD group had slightly lower BDNF concentrations, the global profile of the post-methylphenidate BDNF concentration in the PAD group was very similar to that of the PHI/CD group before treatment (middle and bottom images, Fig. 1); consequently, there were no differences between the ADHD groups after chronic methylphenidate treatment (Fig. 2). Before methylphenidate treatment, the BDNF profile of the PAD group was comparable to that of the control group. Other reports have shown conflicting or opposite results. In particular, there were two reports of

Table 3 BDNF values before and after prolonged release methylphenidate in ADHD groups as a function of the absence/presence of depressive symptomatology

Group		*n* = 148		BDNF (brain-derived neurotrophic factor)											
				Baseline						After PRMPH					
				Depressive symptomatology											
		DS		No (*n* = 123)			Yes (*n* = 25)			No (*n* = 123)			Yes (*n* = 25)		
		No	Yes	D	*N*	Total	D	*N*	Total	D	*N*	Total	D	*N*	Total
CG		41	–	36.36 ± 11.62*	31.78 ± 11.92	34.39 ± 11.88	–	–	–	36.36 ± 11.62	31.78 ± 11.92	34.39 ± 11.88	–	–	–
ADHD	PAD	23	6 (20.7 %)	35.29 ± 11.07§	25.44 ± 9.97¥	30.37 ± 11.53	35.37 ± 20.24	30.31 ± 17.45	32.84 ± 18.01	27.92 ± 10.52€	27.74 ± 9.51	27.83 ± 9.86	26.57 ± 9.93	16.81 ± 10.10	21.69 ± 10.64
	PHI/CD	59	19 (24.4 - %)	28.83 ± 11.47	28.51 ± 11.34	28.66 ± 11.34	35.64 ± 15.20¶	29.24 ± 17.44	32.44 ± 16.42	30.39 ± 12.09	28.70 ± 13.27	29.51 ± 12.66	29.13 ± 14.51	24.66 ± 10.15	26.81 ± 12.43
Total		123	25	32.04 ± 10.54	29.13 ± 12.18	30.61 ± 11.44	35.58 ± 15.98	29.49 ± 17.00	32.54 ± 16.59	31.54 ± 10.37	29.96 ± 13.06	30.77 ± 11.75	28.56 ± 13.40	23.00 ± 10.39	25.71 ± 12.11

Values are expressed as the mean (standard deviation)

PRMPH prolonged release methylphenidate, SD depressive symptoms, CG control group, PAD predominantly attention deficit group, PHI/CD predominantly hyperactive–impulsive/conduct disorder group

Comparisons between groups/subgroups: (*) $z = 1.75$, $p = 0.08$ vs baseline day PHI/CD without DS. ( ) $z = 1.95$, $p = 0.05$ vs baseline day PDA without DS. (¥) $z = 2.05$, $p = 0.04$ vs night CG. (¶) $z = 1.87$, $p = 0.06$ vs basal PHI/CD without DS. (€) $z = 2.24$, $p = 0.025$ vs baseline day PAD group without DS

Day/night fluctuations: (§) $z = 3.55$, $p = 0.0004$ vs night baseline PAD. ( ) $z = 1.86$, $p = 0.06$ vs day baseline PHI/CD with DS. ( ) $z = 3.55$, $p = 0.0004$ vs night PAD without DS. All statistical significances shown were obtained after adjusting for age and sex

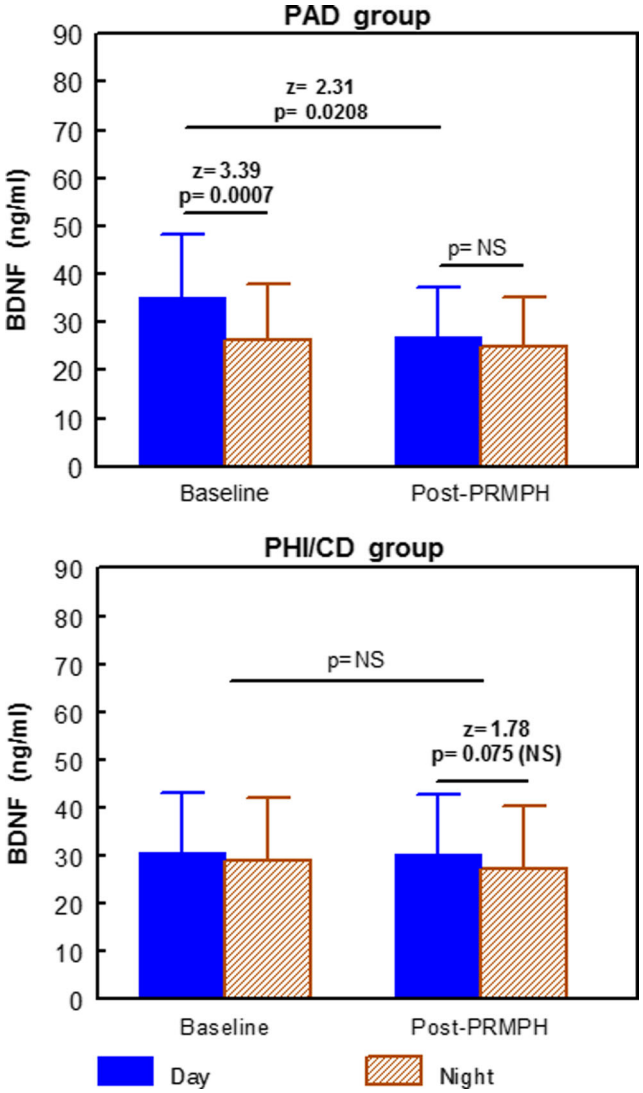

Fig. 2 Comparison of morning BDNF values between ADHD groups (baseline and after chronic treatment with prolonged release methylphenidate). *PAD* predominantly attention deficit group, *PHI/CD* predominantly hyperactive–impulsive/conduct disorder group, *Post-PRMPH* post-prolonged release methylphenidate

untreated children that indicated high BDNF concentrations (Li et al. 2014; Shim et al. 2008), and two other works found no baseline differences (Sahin et al. 2014; Scassellati et al. 2014) compared to the control group. There have been conflicting results for the BDNF changes induced by methylphenidate in children, with only one report indicating a BDNF decrease (Sahin et al. 2014) and another reporting an increase in BDNF after treatment (Amiri et al. 2013).

In experimental developmental animal models, exposure to psychostimulants produced effects that were the opposite of those observed in drug-exposed adult animals (Brandon et al. 2001). During development, exposure to methylphenidate induced brain region-specific decreases in the BDNF gene with a reduction in BDNF messenger RNA (mRNA) in the striatum and the hypothalamus and a permanent reduction in prefrontal BDNF transcription and translation upon cocaine exposure in adulthood (Andersen and Sonntag 2014). Methylphenidate could enhance neurogenesis in the hippocampi of adolescent mice by increasing the BDNF level (Lee et al. 2012). The delayed prefrontal cortex development observed in children and ADHD adolescents could be activated by prepubertal exposure to psychostimulants, which might also be associated with reduced substance abuse disorders as adults (Andersen 2005). By translating this basic research into clinical practice, evidence has emerged that stimulant treatment decreased the risk for subsequent comorbid psychiatric disorders and academic failure (Biederman et al. 2009), but actual use of medications for ADHD did not protect from or contribute to the risk of substance use by adolescents (Molina et al. 2013). It was suggested that prepubertal treatment with a dopamine D3 receptor agonist might reduce ADHD behaviours, including the risk for substance abuse disorders (Andersen and Sonntag 2014).

The postulated predominantly inhibitory changes in brain activity induced by methylphenidate (Volkow et al. 2013) and the role of BDNF in synaptic plasticity should be reflected in the serum BDNF levels. Amiri et al. (2013) found an improvement in hyperactivity symptoms with decreasing baseline plasma BDNF levels. Although their work also reported an increase in BDNF in response to methylphenidate, this increase might reflect the induction of BDNF expression as a downstream response to D1-like dopamine receptor activation (Williams and Undieh 2009). Nevertheless, as suggested above, the changes in brain activity induced by methylphenidate are probably predominantly inhibitory in nature and intermediated by activation of D2 and D3 dopamine receptors (Volkow et al. 2013).

Data reporting differences between ADHD subtypes have been scarce, and no differences at baseline (Scassellati et al. 2014) or in low-level trends in adults have been observed (Corominas-Roso et al. 2013). These latter data were in agreement with the significant, positive correlation between BDNF and the severity of the inattentive symptoms in children (Shim et al. 2008). In addition to a small sample, the differences in the evaluations and classifications of children into subtypes could explain the absence of the detection of differences between subtypes. In our case, as explained in the 'Methods' section, we followed a quantitative method based on EDAH parent scores to separate children into ADHD groups, with the PAD group meeting the DSM-IV criteria for the attention-deficit ADHD subtype and the HI/CD group, which, in addition to patients with the pure hyperactive–impulsive ADHD subtype, included patients with comorbid conduct disorders.

After treatment, we noted a decrease in BDNF only in the PAD group. In some patients, the clinical response to methylphenidate was of lower intensity (Table 2) and resulted in no BDNF changes in the PHI/CD group. Nevertheless, the treatment resulted in the alleviation of clinical symptoms.

Similar to our results with methylphenidate, in adult ADHD subjects treated with atomoxetine, the inattentive group showed a decrease in serum BDNF, while the concentration remained unchanged in the combined ADHD subtype (Ramos-Quiroga et al. 2014). However, it was suggested (Fumagalli et al. 2010) that atomoxetine and MPH exert opposite modulation of the BDNF system, primarily in the prefrontal cortex. The Ramos-Quiroga group previously reported low baseline serum BDNF levels in adults with ADHD compared to controls, suggesting that these low levels might contribute to neurodevelopmental deficits in these patients (Corominas-Roso et al. 2013). In the work of Ramos-Quiroga et al. (2014), the magnitude of the difference in serum BDNF levels between patients and controls was much greater than the change in serum BDNF after chronic treatment with ATX. In our study, this result was not observed because the baseline BDNF concentrations in healthy children and PAD patients did not have any differences, and methylphenidate treatment erased the differences between the ADHD groups, eliminating the statistically significant differences between the control and the PHI/CD group. It is noteworthy that the clinical efficacies of both stimulant and non-stimulant treatments for ADHD have been associated with similar decreases in BDNF concentration. Hypothetically, long-term effects (i.e. neural circuitry reorganisation or neuronal plasticity) induced by ADHD medications previously administered to adult patients (Ramos-Quiroga et al. 2014) might have induced a prolonged and progressive decrease in BDNF levels, which would explain the greater difference between inattentive adult patients and control patients than those observed for the group of predominantly inattentive children.

Although ADHD has a strong genetic basis (Biederman and Faraone 2005), several meta-analyses of reported differences in serum BDNF levels in ADHD patients have not found any evidence for differences in the frequency of BDNF genotypes in ADHD (Sanchez-Mora et al. 2010; Zhang et al. 2012). In addition to these yet undefined genetic influences, a variety of stressful events experienced during pregnancy predicted ADHD (Ronald et al. 2010). These events could act as epigenetic mechanisms at the interface between BDNF polymorphisms and ADHD symptoms (Lasky-Su et al. 2007). For example, smoking induces both significant decreases in striatal and cortical dopamine and serotonin and reductions in BDNF mRNA and proteins, resulting in the long-term downregulation of BDNF expression (Toledo-Rodriguez et al. 2010). These data were in agreement with the behavioural alterations observed in epidemiological studies linking maternal smoking to ADHD. Another toxic factor, prenatal alcohol exposure, could affect both BDNF expression and neurogenesis via epigenetic mechanisms (Ungerer et al. 2013). Although the neuropsychological effects of alcohol exposure in intrauterine life might be clinically differentiated from those in children with ADHD (Mattson et al. 2013), an exacerbated relationship between alcohol exposure and ADHD in conduct disorders and externalising behavioural problems in children has been shown (Ware et al. 2014). In addition to reduced morning serotonin concentrations (the entire ADHD group), ADHD children in the PHI/CD group showed reduced baseline serum BDNF (Molina-Carballo et al. 2013). Innately, low BDNF expression due to genetic polymorphisms or other causes could play a role in the mediation of the reinforcing effects of ethanol and the control of ethanol intake (Raivio et al. 2014) at later ages, in turn accentuating serotonin system dysfunction (Riikonen et al. 2005). BDNF exerts trophic properties on the serotonin neurons; that is, in animal models of foetal alcohol syndrome and prenatal stimulant (cocaine) administration, activation of the serotonin-1A receptor induced the growth of atrophic neurons and reversed microcephaly, a key characteristic of alcohol-related foetal spectrum disorders (Azmitia 2001).

The decreased serum BDNF levels in the ADHD group and more specifically in the PHI/CD group, as well as in the adults with ADHD (lower BDNF levels in the combined group than in the inattentive subtype) (Corominas-Roso et al. 2013), appeared to contradict the results of Shim et al., who reported significantly higher BDNF levels in ADHD children than in controls. These authors suggested a compensatory mechanism in the response of abnormal and late brain maturation in ADHD children (Shim et al. 2008). Corominas-Roso et al. suggested (2013) that patients whose ADHD persisted into adulthood could be a subgroup with lower intrinsic BDNF activity, which might contribute to maintaining the disorder.

One of the most frequent confounding factors when studying serum BDNF levels is the presence of depressive symptoms. Depression has been consistently associated with a decrease in BDNF levels in the serum (Bus et al. 2011), which is normalised with antidepressant treatment. We found no influence of BDNF on depressive symptomatology in children. Our study protocol did not specifically exclude the presence of anxiety symptoms, although low BDNF levels have been reported in female patients with anxiety disorders (Molendijk et al. 2012). Although we reported statistical data adjusted for age and sex, other authors have shown no sex differences in serum BDNF levels in adult ADHD patients. Together, these data further suggested that low serum BDNF levels are a characteristic of a subset of ADHD patients.

These results for BDNF were consistent with the previous work of our group (Muñoz-Hoyos et al. 2011) and of others (Girdler and Klatzkin 2007), suggesting that low basal neuroendocrine mediator levels in response to chronic stress and inadequate responses to stimuli are the consequences of repeated biological adaptations to increased life stress. Some neurosteroids evoke this type of response (Molina-Carballo et al. 2014). Neuroactive steroids can be synthesised in neuronal and glial cells independent of peripheral steroidogenesis. These steroids have modulatory effects on the release of multiple neurotransmitters. Furthermore, they have important functions in

development (Melcangi et al. 2014). Among them, dehydroepiandrosterone has been suggested to be a biological marker for ADHD (Wang et al. 2011). In contrast, the published data on the interactions among serotonin, dehydroepiandrosterone and BDNF (Martinowich and Lu 2008) have included BDNF as a mediator of DHEA activity in the brain, resulting in a significant increase in serotonin levels after DHEA administration (Svec and Porter 1997). Allopregnanolone, another key neurosteroid derived from progesterone, has a low serum concentration during depressive episodes in humans with a blunted increase in response to acute stress (Girdler and Klatzkin 2007). In agreement with these data, methylphenidate induced a significant increase in serum allopregnanolone only in PAD–ADHD children without depressive symptoms, while in the PHI/CD group, it induced a decrease, which had statistical significance only in the presence of depressive symptoms (Molina-Carballo et al. 2014). Finally, sex differences in the expression and/or responses to neurosteroids of the nigrostriatal dopaminergic system (Gillies et al. 2014) have been proposed to explain the gender differences in the incidence of ADHD. Steroidogenic antidepressants could increase allopregnanolone synthesis (Evans et al. 2012), which, in turn, might upregulate the BDNF content in the cortico-limbic neurons, exerting trophic effects (Nakao et al. 2011).

The daily fluctuations in BDNF showed significant day/night fluctuations with higher morning values and no significant changes after treatment. Neuroendocrine changes in ADHD might involve disturbances to various aspects of the biological rhythms that normally exhibit circadian oscillations (Kohyama 2011). Irregular sleep–wake patterns and delayed sleep in individuals with ADHD were associated with delays and dysregulations of the core and skin temperatures (Bijlenga et al. 2013). We previously reported that subtle changes in the daily fluctuations and concentrations of serotonin and melatonin (Molina-Carballo et al. 2013) and melatonin metabolisation (Cubero-Millan et al. 2014) might contribute to marked clinical improvements in the key symptoms of ADHD.

Considering the BDNF decrease in morbidly obese children, the co-occurrence of both obesity and ADHD (Cortese et al. 2013) and the role of BDNF in cognition (Carlino et al. 2013), further phenotypical studies on the concentrations of BDNF in children and adults are warranted. Our patients and those of Scassellati et al. (2014) had similar BMI values for both the ADHD subtypes and the controls. Future studies on BDNF and other neuroendocrine mediators of ADHD in humans should correct for the time of blood withdrawal, age, sex, sociodemographic variables, smoking status and food and alcohol intake, and these studies should stratify ADHD patients according to their BMI so that several other ADHD subclassifications can be defined based not only on symptomatology but also on biology (Wallis 2010).

Our study, which reported the objective neuroendocrine responses after chronic treatment, had several limitations as follows: (1) the study included a small control group largely composed of the siblings of the patients. Nevertheless, our sample might be homogeneous with respect to the genetic and epigenetic variables related to the expression of BDNF and other variables that could alter BDNF levels, because a high-fat diet (Kaczmarczyk et al. 2013) and exercise (Heyman et al. 2012) rapidly impact dopamine metabolism in the brain. (2) There were small numbers of females, adolescents and patients belonging to the PAD group. (3) A large proportion of the ADHD children had comorbid CD.

In summary, our results showed both a decrease in the baseline serum BDNF levels of children with ADHD, due to a lower value in predominantly hyperactive–impulsive/conduct disordered children, and that methylphenidate treatment eliminated these baseline differences in BDNF concentrations between ADHD groups by lowering BDNF in the PAD group. Despite region-specific differences in BDNF expression and synaptogenesis during brain development (Ninan 2014), if decreases in BDNF serum concentrations are a common feature of a subset of children and adult ADHD patients, it must be confirmed with additional studies including larger samples and groups with homogeneous semiology.

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Compliance with ethical standards

The study design and outcome variables were approved by the Hospital Ethics Committee and the Health Research Fund of the Spanish Ministry of Science and Innovation.

Contributors AMC, JU and AMH designed the study and wrote the protocol. ICM, MJRR, SMS, LFL, PTP, ARL and IMC performed the sample collection and managed the literature searches and analyses. AMC, AMH and JDLC undertook the statistical analysis; and ICM, MJRR and AMC wrote the first draft of the manuscript. All of the researchers contributed to and have approved the final manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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