Psychopharmacology
DOI 10.1007/s00213-014-3514-5

ORIGINAL INVESTIGATION

Differential responses of two related neurosteroids to methylphenidate based on ADHD subtype and the presence of depressive symptomatology

Antonio Molina-Carballo · Fuensanta Justicia-Martínez · Francisco Moreno-Madrid · Isabel Cubero-Millán · Irene Machado-Casas · Laura Moreno-García · Josefa León · Juan-de-Dios Luna-del-Castillo · José Uberos · Antonio Muñoz-Hoyos

Received: 18 November 2013 / Accepted: 15 February 2014

Abstract
Rationale Attention deficit with hyperactivity disorder is a neurodevelopmental disorder associated with alterations in the prefrontal cortex via dopaminergic and noradrenergic neurotransmission. Neurosteroids (e.g. allopregnanolone and dehydroepiandrosterone) modulate the release of multiple neurotransmitters.
Objective This study aims to determine the baseline concentrations and daily variations in allopregnanolone and dehydroepiandrosterone in children with attention deficit hyperactivity disorder (ADHD) and to determine the effect of chronic administration of methylphenidate on clinical symptoms and on the concentrations of these two neurosteroids.
Methods We included 148 children aged 5 to 14 years, subdivided into two groups: ADHD group (n=107, with a diagnosis of ADHD (DSM-IV-TR criteria), further classified in subtypes by an "attention deficit and hyperactivity scale" and subgroups by the "Children's Depression Inventory") and a control group (n=41). The clinical workup included blood samples that were drawn at 20:00 and 09:00 hours, at inclusion in both groups, and after 4.61±2.29 months of treatment only in the ADHD group, for measurements for allopregnanolone and dehydroepiandrosterone. Factorial analysis, adjusted for age and gender, was performed by using Stata 12.0.
Results Methylphenidate induced the doubling of allopregnanolone levels in the predominantly inattentive ADHD patients without depressive symptoms (27.26±12.90 vs. 12.67±6.22 ng/ml, morning values). Although without statistical differences, baseline dehydroepiandrosterone levels were higher and slightly increased after methylphenidate in the ADHD subtype with depressive symptoms (7.74±11.46 vs. 6.18±5.99 ng/ml, in the morning), opposite to the lower baseline levels, and further decrease after methylphenidate in the inattentive subtype with depressive symptoms.
Conclusions Different neurosteroids may have different baseline concentrations and differential responses to methylphenidate treatment as a function of ADHD subtype and subgroup. These differential responses may be a clinical marker of ADHD subtype and/or co-morbidities.

Keywords ADHD · ADHD subtypes · Depressive symptoms · Children · Prolonged release methylphenidate · Neurosteroids · Allopregnanolone · Dehydroepiandrosterone

A. Molina-Carballo · F. Justicia-Martínez · I. Cubero-Millán · I. Machado-Casas · L. Moreno-García · A. Muñoz-Hoyos
Servicio de Neuropediatría, Neuropsicología y Atención Temprana, Unidad de Gestión Clínica de Pediatría, Hospital Clínico San Cecilio, Complejo Hospitalario Granada, Granada, Spain

J. León
FIBAO, Hospital Clínico San Cecilio, Junta de Andalucía, Spain

J.-d.-D. Luna-del-Castillo
Departamento de BioEstadístic, Facultad de Medicina, Universidad de Granada, Granada, Spain

F. Moreno-Madrid · J. Uberos
UGC Pediatría, Hospital Clínico San Cecilio, Avda Dr. Olóriz 16, Granada, Spain

A. Molina-Carballo (✉) · F. Moreno-Madrid · J. Uberos · A. Muñoz-Hoyos (✉)
Departamento de Pediatría, Facultad de Medicina, Universidad de Granada, Avda de Madrid 12, 18012 Granada, Spain
e-mail: amolinac@ugr.es
e-mail: amunozh@ugr.es

Introduction

The theories about the neurobiological basis of attention deficit hyperactivity disorder (ADHD) have been recently centred

Published online: 06 March 2014

on two complementary models (Barkley 1997; Nigg 2005), both of which are based in the dysregulation of interacting neural pathways, i.e. the inhibitory noradrenergic frontocortical activity on dopaminergic striatal structures (Wiltschko et al. 2010) and the ascending dopamine circuits besides the limbic system (Nigg and Casey 2005). As a neurodevelopmental disorder, in ADHD, there are age-related changes in discrete brain volume areas and connectivity (van Ewijk et al. 2012) that parallel behavioural improvement and increased efficiency in cognitive task performance (Gupta and Kar 2009; Matthews et al. 2013).

Neuroactive steroids may be synthesised in neuronal and glial cells independently from the peripheral steroidogenesis occurring in endocrine glands and have autocrine and paracrine actions in the central nervous system (Compagnone and Mellon 2000). At the cellular level, in addition to their impact on postsynaptic receptors, they have modulatory effects on the release of multiple neurotransmitters, including dopamine, norepinephrine and serotonin (Zheng 2009). Allopregnanolone (ALLOP) and dehydroepiandrosterone (DHEA) directly stimulate catecholamine secretion by inducing tyrosine hydroxylase (Charalampopoulos et al. 2005).

ALLOP promotes the following: (1) NMDA-induced striatal dopamine release (Kretschmer 1999), (2) noradrenaline release in the cerebral cortex (Puia et al. 2012), (3) proliferation of human neural progenitor cells via GABA-A receptor-activated voltage (Jagasia et al. 2009) and (4) inhibition of glutamate release in the prefrontal cortex (Moghaddam et al. 1990). In addition, in experimental models, basal forebrain and brainstem levels of ALLOP regulate the sleep/wake cycle as well as sleep-dependent memory impairment (George et al. 2010).

DHEA sulfate is desulfated enzymatically to produce DHEA, which is, in turn, converted into various estrogenic and androgenic compounds. The two forms are easily interconverted. DHEA easily crosses the blood–brain barrier and is highly concentrated in the brain (Traish et al. 2011). DHEA has multiple effects on neuronal survival, brain development and cognition and protects against the apoptotic loss of dopaminergic neurons (Charalampopoulos et al. 2008). DHEA, by increasing glutamate release, can strengthen physiological glutamatergic tone, consequently improving memory in inhibitory avoidance tasks.

The key features of ADHD include the presence of the core problems of inattention, hyperactivity and impulsivity. Additionally, the vast majority of ADHD patients have at least one co-morbid condition, e.g. conduct disorder, depressive symptoms or sleep disorders. While both ADHD subtypes had depressive symptom severity equal to a non-ADHD psychiatric control group and greater than community control groups, externalizing behaviour problems and aggression appeared to be related to the hyperactive–impulsive ADHD symptom domain and to the overall ADHD symptom severity (Connor and Ford 2012).

The aim of this study is to examine the relationship between blood levels of DHEA and ALLOP as well as their daily fluctuations in ADHD children prior to and after chronic methylphenidate treatment; in addition, the relationship of these levels to clinical symptomatology is evaluated to determine whether these neuroendocrine mediators actively participate in the pathophysiology of ADHD or the response to ADHD treatment.

Material and methods

Subjects and samples

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 that primarily report objective neuroendocrine measures of response.

The sample consisted of two groups: an ADHD group in which each included patient was assessed at least twice and consequently may be considered as his/her own control and a control group as a reference. A total of 107 children who met the DSM-IV-TR/ICD-9 criteria for ADHD (American Psychiatric Association 2002) were included in the ADHD group after completing the clinical protocol in order to exclude the main co-morbidities. Upon the inclusion of an ADHD patient in the study, we included a control subject (n=41). Siblings (recruited simultaneously to his/her brother) or unrelated subjects were healthy, and all had adequate academic performance.

Clinical method

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) EDAH scale (Spanish acronym for evaluation of deficit of attention and hyperactivity scale) (Sánchez et al. 2010; Zambrano-Sánchez et al. 2011), in duplicate, one for the teacher and the other for the child's parents; (c) the Children's Depression Inventory (CDI), which was completed by subject aged ≥8 years; and (d) a sleep diary that 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 a structured observation by teachers and is divided into two ten-item subscales for ADHD and CD. Based on EDAH, the ADHD group was subclassified into two clinical subtypes: children with predominantly attention deficit [PAD; if attention deficit (AD) >9, hyperactivity–

impulsivity (HI) <10 and total scores <30] and children with predominantly hyperactive–impulsive/conduct disorder (PHI/CD; if AD <10, hyperactivity (H) >9 and/or total punctuation >29). Therefore, of the 78 children who were included in the PHI/CD group, 34 of them (44 %) met criteria for the diagnosis of HI without CD. Of the 44 children with symptoms of CD, 33 showed a predominance of symptoms of HI on the symptoms of CD, while the rest of the children (11/78; 14 %) had a prevalence of symptoms of CD on the symptoms of HI. Only 26 of 78 children in this group (33 %) did not meet further criteria for attention deficit.

The d2 test (Brickenkamp 1997) is a measure of attention, particularly visual attention. d2 measures processing speed, rule compliance and quality of performance, allowing for a neuropsychological estimation of individual attention and concentration performance, by quantification of 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 (Kovacs 1992) is a self-report assessment of depression for children whose two subscales (negative mood and negative self-esteem) consist of the items that are most unique to depression and least related to anxiety. For defining subgroups, we considered the sum of both subscales, with a cut-off of >17 points considered pathological.

All children were evaluated using an abbreviated intelligence test as a screening cognitive ability [KBIT (Kaufman and Kaufman 1996)] and also completed the Spanish version of the sleep diary of the National Sleep Foundation for 1 week, and the ADHD group completed the diary once again after the treatment.

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

The exclusion criteria were (1) KBIT <85, (2) pre-existing 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 according to function of 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 patients were naïve of any medication, and no other treatment (pharmacological or psychological) was administered before conclusion of the protocol.

Table 1 Somatometrics, vital signs as well as haematological, biochemical and sleep data for the study groups at inclusion in the protocol

	Control (n=41)	ADHD (n=107)	Statistics	
			t	p
Age (years)	10.22±2.58	9.39±2.5	1.81	0.07
Sex (M/F)	30/11	85/22	X^2=0.67	0.41
Height (m)	1.47±0.18	1.37±0.17	3.06	0.001**
Weight (kg)	44.179±15.14	36.50±15.35	2.39	0.003**
BMI (kg/m^2)	19.8±4.14	18.8±4.23	1.14	0.254
HR (bpm)	79.81±12.96	78.94±10.63	0.37	0.71
SBP (mmHg)	105.42±13.80	101.60±13.56	1.41	0.16
DBP (mmHg)	64.11±8.57	64.607±13.27	0.241	0.81
Hb (g/L)	13.87±1.02	13.94±0.78	0.40	0.69
Hct (%)	39.18±2.40	39.63±6.42	0.39	0.70
MCV (fl)	78.16±8.90	78.84±8.95	0.37	0.71
Iron (mg%)	84.68±29.76	85.29±31.27	0.10	0.92
Ferritin (ng/L)	38.12±13.53	42.05±19.74	1.07	0.29
TSH (uUI/L)	2.44±1.26	2.93±1.374	1.77	0.08
SOD	None	30 (23.07 %)	–	–
Enuresis	None	16 (12.30 %)	–	–
KBIT score	107.88±12.29	103.99±11.23	0.777	0.210

Data are expressed as mean±SD

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, *SOD* sleep onset delay (<60 min in all subjects), *MCV* mean corpuscular volume, *TSH* thyroid-stimulating hormone, *KBIT* combined punctuation of the Kaufman abbreviated intelligence test

** p<0.01

Measurements None of the samples were obtained in the presence of an acute or severe illness. Blood samples were taken at 20:00 and 09:00 hours the following day. 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 method

DHEA was measured using the RIA Kit DSL-8900 (Catalog Number: DSL-8900) with a detection range of 0.2–30 ng/mL and a sensitivity of 0.01 ng/mL. For human ALLOP, we used an ELISA kit (E91963Hu, USCN), which has a detection range between 1.23 and 100 ng/mL and a minimum detectable dose (sensitivity) of less than 0.44 ng/mL.

Statistical method

To achieve the objectives of the study, factorial analyses were conducted as described below. For comparisons between 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) subtype with two categories (PAD and PHI/CD subtypes); (b) patients, nested in subtypes and subgroups (CDI); (c) hour, with two categories (day and night), and crossed with subtype; and (d) time, with two levels before and after treatment. This factor was a crossed factor with subtype and hour. Subtype, hour and time were fixed effects factors, and patients was a random effects factor. Comparisons between cases and controls were performed because there was only one measure for controls using the same analysis repeated in two different situations, which are 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) that was crossed with group. Group and hour were fixed effects factors, and subjects were a random effects factor. For both types of comparisons, an ANOVA table was built, and higher interactions were determined. If these were significant, multiple pairwise comparisons were made 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 have used "*z*", the normal approximations for "*t*s", because of global sample sizes. The analyses reported were crude analyses, and adjusted analyses by age and gender were carried out using ANCOVA methodology. In all cases, the interactions were studied for levels below 0.15, and the latest comparisons were considered significant at $p<0.05$ after applying the penalty provided by the correction. When analysing the variances in different groups, homogeneous transformations were carried out on data using natural logarithm to achieve uniformity. We used the statistical package Stata 12.0 for all analyses.

Results

The mean height and weight was significantly higher in the control group, in part due to the slightly higher mean age than that noted in the ADHD group, without differences in BMI (Table 1). After treatment, the increase in height for patients was unaffected, while weight decreased, which was expected and previously reported (Molina-Carballo et al. 2013). There were no significant changes in the vital signs or the haematological/biochemical variables measured.

Table 2 shows clinical course data (EDAH, d2 and CDI scores) for the ADHD group, separated into diagnostic subtypes. Although not significantly different, the incidence of depressive symptoms was 20.7 and 24.4 % in the PAD and PHI/CD subtypes, respectively, and was more common between girls than boys (34.8 vs. 20.2 %, respectively, in the whole ADHD sample). More than 80 % had improvement in parent evaluation data after methylphenidate, with almost one third of participants reporting scores on patient evaluation data that were similar to those of control subjects.

Allopregnanolone

Comparisons between groups and subtypes At baseline, children with ADHD (12.9±10.63 ng/ml) had significantly ($z=2.15$, $p=0.023$) lower concentrations of ALLOP than the control children (14.64±10.9 ng/ml). After adjusting for age and gender, these significant differences were erased. In both the control group and the ADHD group, there was no daily fluctuation in ALLOP concentration. Only slightly higher ALLOP values were found in the morning than in the evening in the control group (14.64±10.9 vs. 10.84±4.67 ng/ml).

There were no differences in the baseline ALLOP levels between the control group and ADHD subtype groups as well as between control group and ADHD subtypes when subdivided according to the presence or absence of depressive symptomatology. This is also true when comparing daily fluctuations of ALLOP levels between subtypes/subgroups, with the exception of significant day/night differences in PHI/CD patients with depressive symptoms, due to both high morning values, with the lowest ALLOP values occurring at night ($z=3.52$, $p=0.0004$).

ALLOP response in whole ADHD group After treatment, ALLOP levels showed a very significant increase both in the morning ($z=3.49$, $p<0.00001$) and at night ($z=2.6$, $p=0.0093$), without significant differences in the response between the day and night, except for ADHD children without depressive symptomatology due to a much higher morning response than at night ($z=2.03$, $p=0.04$) in this subgroup. The absence of depressive symptoms was indicative of high ALLOP increases after methylphenidate ($z=3.03$, $p=0.025$, vs. the presence of depressive symptoms).

ALLOP response by ADHD subtypes ALLOP response (Table 3) was very different between the PAD and PHI/CD subtypes, with a very significant increase in ALLOP only for the PAD subtype without depressive symptomatology, both during the day ($z=5.02$, $p<0.00001$) and at night ($z=2.64$, $p=0.0083$) (Fig. 1), without significant differences in levels between day and night. The presence of depressive symptomatology completely erased and even induced an opposite

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

Test	Score	Control group	ADHD group							
			PAD subtype				PHI/CD subtype			
			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* Childhood Depression Inventory, *NSE* value of the "negative self-esteem" subscale of the CDI, *Z Z* value on the Wilcoxon signed-rank test, *NS* not significant, *PRMPH* prolonged release methylphenidate

[a] Statistical comparison in the ADHD subgroup between baseline and post-PRMPH data

response of ALLOP to methylphenidate in PAD–ADHD children.

In addition, in the PHI/CD subtype without depressive symptoms, there were slight non-significant morning increases in ALLOP levels after methylphenidate (p=0.054), with no change at night.

In contrast, both ADHD subtypes with depressive symptoms showed a decrease in morning ALLOP concentration, which was significant only for the PHI/CD subtype (p=0.04) with a slight non-significant increase at night only for the PHI/CD subtype (Table 3).

Dehydroepiandrosterone

Comparisons between groups and subtypes DHEA concentrations were higher in the control group (day, 6.43±6.78 ng/ml; night, 4.34±3.03 ng/ml) with significant differences in the ADHD group for both daily comparisons (z=13.57, p<0.001, during the day, and z=10.6, p<0.001, during the night), although these differences disappeared after adjustment.

Adjusted DHEA levels showed a significant daily variation with greater concentrations in the morning in both study groups (z=−2.12, p=0.034, for control group; z=2.2, p=0.027, for ADHD group).

Baseline comparisons of the means of the control group with ADHD-subtype groups (PAD, day/night, 5.48±4.63/3.6 ±3.04 ng/ml vs. PHI/CD, day/night, 4.5±4.79/3.71± 2.86 ng/ml) showed no differences in DHEA concentration between control group vs. PAD subtype, nor between PAD vs. PHI/CD and only a non-significantly greater value of DHEA in control group vs. PHI/CD subtype (p=0.07).

DHEA response for the entire ADHD group For the entire ADHD group, methylphenidate treatment did not induce any changes in DHEA concentrations, during the day (before vs. after, 4.76±4.74 vs. 5.17±6.82 ng/ml) or at night (3.68±2.89 vs. 3.59±2.83 ng/ml); thus, the high, significant morning predominance persisted (z=2.85, p=0.004).

DHEA response by ADHD subtypes DHEA response to methylphenidate was not different between ADHD subtypes (PAD, day/night, 4.71±3.07/3.49±1.74 ng/ml vs. PHI/CD, day/night, 5.31±7.62/3.62±3.12 ng/ml), although DHEA in the PAD subtype tended to decrease, while the mean value of DHEA in the PHI/CD subtype tended to increase. Methylphenidate treatment induced the highest differences between day and night levels (z=3.72, p=0.0002).

The response of DHEA to methylphenidate in ADHD subtypes showed no significant differences based on depressive symptoms, both in the morning and at night (Fig. 2). This absence of significant differences between the two subgroups prevented multiple pairwise comparisons. The mean±SD

Table 3 Allopregnanolone and dehydroepiandrosterone values both before and after prolonged release methylphenidate (PRMPH) in ADHD subtypes as a function of the absence/presence of depressive symptomatology (DS)

Group		*n*=148		Allopregnanolone							
				Depressive symptomatology							
				Baseline				After PRMPH			
		DS		No (*n*=123)		Yes (*n*=25)		No (*n*=82)		Yes (*n*=25)	
		No	Yes	*D*	*N*	*D*	*N*	*D*	*N*	*D*	*N*
CG		41	–	13.84±10.53	10.84±8.81	–	–	–	–	–	–
ADHD[abc]	PAD	23	6 (20.7 %)	12.67±6.22	13.33±10.23	8.8±3.6	8.1±6.33	27.26±12.90[d]	21.46±15.05[e]	6.19±1.79	7.23±1.40
	PHI/CD	59	19 (24.4 %)	13.00±14.00	12.52±10.31	15.08±9.01	8.95±5.38	15.75±8.94	14.04±11.88	8.89±3.82[f]	12.38±11.05

Group	Dehydroepiandrosterone							
	Depressive symptomatology							
	Baseline				After PRMPH			
	No (*n*=123)		Yes (*n*=25)		No (*n*=82)		Yes (*n*=25)	
	D	*N*	*D*	*N*	*D*	*N*	*D*	*N*
CG	6.43±6.78	4.34±3.03	–	–	–	–	–	–
ADHD[abc]	5.94±5.16	3.88±3.35	3.78±1.65	2.51±0.94	5.47±3.00	3.81±1.79	2.38±2.62	2.05±0.82
	4.24±4.67	3.51±1.58	6.18±5.99	4.74±5.23	4.89±6.31	3.52±2.86	7.74±11.46	4.00±4.00

Values are means (standard deviation). Statistical significances shown are obtained after analysis adjusted by age and sex

CG control group, *PAD* predominantly attention deficit subtype, *PHI/CD* predominantly hyperactive–impulsive/conduct disorder subtype

[a] Day/night fluctuation: $z=-2.12$, $p=0.034$, vs. day control group

[b] Day/night fluctuation: $z=2.85$, $p=0.004$, for baseline whole ADHD group

[c] Day/night fluctuation: $z=3.72$, $p=0.0002$, for whole ADHD group post-PRMPH

[d] $z=5.02$, $p<0.00001$, vs. PAD day

[e] $z=2.64$, $p=0.0083$, vs. PDA night

[f] $z=2.02$, $p=0.0437$, vs. PHI/CD day with depressive symptomatology

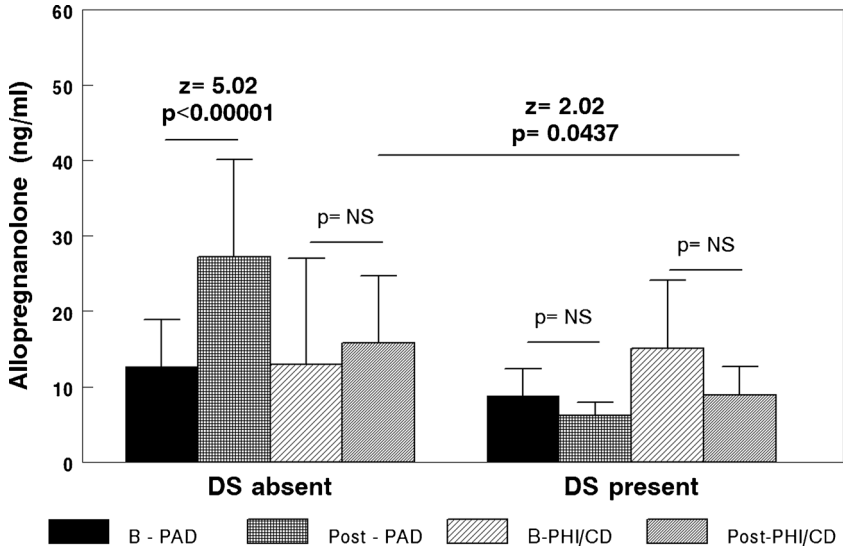

Fig. 1 Comparison of morning allopregnanolone serum levels between ADHD subtypes (baseline and after chronic treatment with prolonged release methylphenidate) with respect to the presence or absence of depressive symptoms. *DS* depressive symptomatology, *PAD* predominantly attention deficit subtype, *PHI/CD* predominantly hyperactive–impulsive/conduct disorder subtype, *B* baseline, *Post* post-PRMPH

values of each subtype and subgroup (Table 3) suggest a trend toward different baseline levels and an opposite response between subgroups. At baseline, the PAD subtype had lower DHEA concentrations, with higher baseline DHEA values for the PHI/CD subtype, both in the presence of depressive symptoms. The response to methylphenidate also seemed to be different, with a decrease in DHEA for both subgroups of the PAD subtype, and an increase in both subgroups of the PHI/CD subtype. The highest DHEA levels were observed in the presence of depressive symptoms.

Discussion

Our study is the first to report ALLOP concentrations among children diagnosed with ADHD, both before and after chronic prolonged release methylphenidate treatment. In addition, we report data for DHEA, which expands the data already published on this neurosteroid (Maayan et al. 2003; van Goozen et al. 2000; Wang et al. 2011). Very few clinical investigations have associated the behaviour of these neurosteroids in children (Fadalti et al. 1999; Grosso et al. 2011; Predieri et al. 2007). Our data reinforce the need to quantify ADHD comorbidities, since this can help to better define the profile of the patient and thus adapt the treatment protocol to the patient's needs and to reformulate the family expectations.

Levels of both neurosteroids were higher in the control group; however, levels of ALLOP stayed higher after adjusting for age and gender only when compared to those of ADHD group without depressive symptoms, while the difference in DHEA levels disappeared after adjusted analysis. Our data indicate that fewer ADHD symptoms (EDAH scale) and fewer depressive symptoms (CDI) after methylphenidate may be related in part to the neuroendocrine changes

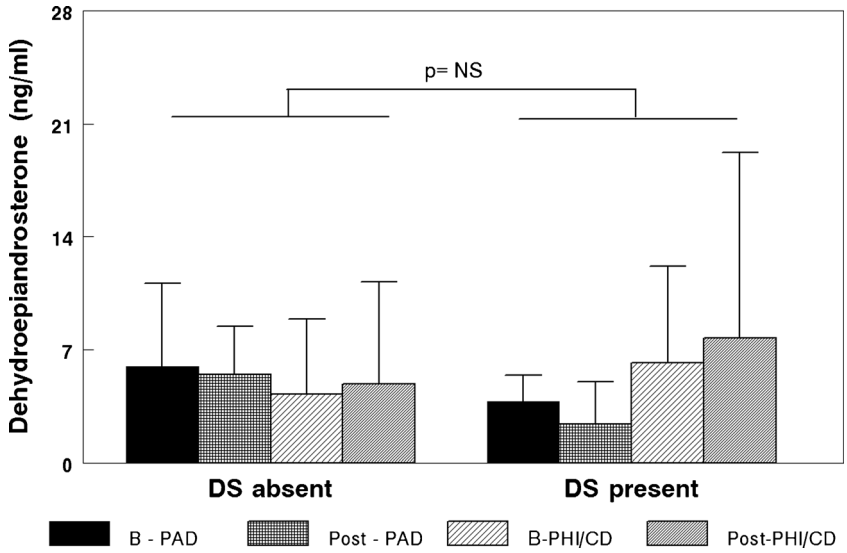

Fig. 2 Comparison of morning dehydroepiandrosterone values between ADHD subtypes (baseline and after chronic treatment with prolonged release methylphenidate) with respect to the presence or absence of depressive symptoms. *DS* depressive symptomatology, *PAD* predominantly attention deficit subtype, *PHI/CD* predominantly hyperactive–impulsive/conduct disorder subtype, *B* baseline, *Post* post-PRMPH

documented in this study. In addition, subtle changes in the daily fluctuations and concentrations of these and other mediators of both serotonin and melatonin (Molina-Carballo et al. 2013) may contribute to marked clinical improvement in the key symptoms of ADHD.

ALLOP is one of the endogenous regulators of seizure susceptibility, anxiety, and stress (Amin et al. 2006; Molina-Carballo et al. 2007; Pluchino et al. 2009). As ALLOP increases during puberty (Predieri et al. 2007), the increasing incidence of depressive symptoms with age might be related to the slightly higher (non-significant) ALLOP levels in our ADHD patients with co-morbid depressive symptomatology or, alternatively, to the increasing incidence of the PAD subtype with increasing age. Both hypotheses are unlikely because depression has been associated with lower levels of ALLOP and to blunted ALLOP responses to stress (Girdler and Klatzkin 2007), and our PAD patients had the lowest ALLOP values. Reports on ALLOP levels in depression are partially consistent with our data because in the presence of depressive symptoms, ALLOP concentrations seem to be different between ADHD subtypes. Only the PAD subtype with depressive symptoms had a lower baseline concentration of ALLOP and a decreased level of ALLOP (but not a blunted) in response to methylphenidate appeared to occur in the PHI/CD subtype and in both subtypes in the presence of depressive symptomatology (DS). The decrease in ALLOP in response to methylphenidate was statistically significant for only the PHI/CD subtype with DS.

Although ALLOP levels have been reported to be low during depressive episodes (Miller and Miller 2001), during euthymia, plasma concentrations of ALLOP in women with bipolar disorders (BPDs) are elevated compared to healthy controls and women with major depressive disorder (MDD) (Bessa et al. 2009). Our patients with PHI/CD subtype had baseline ALLOP levels very similar to the control group irrespective of the presence/absence of depressive symptoms. Methylphenidate induces a very significant increase in the concentration of ALLOP in PAD patients without co-morbid depressive symptoms only.

A decrease in brain ALLOP is associated with induced aggressive behaviour, and ALLOP administration has been implicated in suppressing irritability, which is a component of mood elevation in bipolar disorder (Pinna et al. 2005). Johannson et al. (2011) found that in males with bipolar disorder, progesterone concentrations were lower in those who had shown manic/hypomanic irritability compared with non-irritable patients. BPD and ADHD share risk factors, distinct subtypes and weak causal relationships, and for many researchers, type I BPD plus co-morbid ADHD is a distinct familial subtype of either type I BP disorder or ADHD (Biederman et al. 2013) because a bidirectional and robust co-morbidity between paediatric bipolar disorder and ADHD has been documented. On average, 62 % of patients with BPD also meet criteria for ADHD. The co-occurrence of ADHD and BPD is associated with poorer global functioning, greater symptom severity and additional co-morbidity than for either disorder individually (Kowatch et al. 2005).

Similar to other neuroendocrine mediators (Muñoz-Hoyos et al. 2011) while neurosteroid increases in response to stress are adaptive in the short term, animal models of chronic stress and depression found lower brain and plasma neurosteroid concentrations and alterations in neurosteroid responses to acute stressors. These results are consistent with ours related to low basal neurosteroid levels with the absence of an adequate response to stimulus (i.e. decreases in ALLOP to methylphenidate in the PAD–ADHD subtype if a co-morbid condition such as depression exists). This failure to mount an appropriate ALLOP response to stress may reflect the price of repeated biological adaptations to increased life stress, and altered ALLOP stress responsivity may also contribute to the dysregulation observed in hypothalamic–pituitary–adrenal (HPA) axis function in depression (Girdler and Klatzkin 2007). The effect of ALLOP on dopamine release in the prefrontal cortex may play a role in connecting the change in ALLOP concentrations and an altered emotional state (George et al. 2010).

It has been suggested that ALLOP, which has biphasic effects at low concentrations, potentiates an anxiogenic effect on patients, whereas higher ALLOP concentrations decrease this effect and show beneficial, calming properties. A subset of treated ADHD children also experience negative mood symptoms, and the neuroendocrine basis of this paradoxical effect is unclear. The dual and opposite changes in neurosteroids reported in this study may account for some of these paradoxical effects. ALLOP treatment in women increased the activity in the amygdala in a similar way to the changes observed during anxiety reactions (Andreen et al. 2009).

The male bias in the prevalence of ADHD may be related to steroid sulfatase, an enzyme encoded by an X-linked gene responsible for the synthesis of DHEA from DHEA-S (Brookes et al. 2008). Similar to our data, experimental (Trent et al. 2013) and clinical studies (Strous et al. 2001) have reported significant inverse correlations between clinical symptomatology (in particular, hyperactivity symptomatology) and DHEA levels. In addition, morning DHEA salivary levels were correlated with composite scores of distractibility and impulsivity on continuous performance test. All these papers conclude that DHEA, as well as other neurosteroids, may be a biological laboratory marker for ADHD (Wang et al. 2011).

Neurosteroid homeostasis may be critical for normal cognitive development (Yang et al. 2009), neurocognitive performance (Galderisi et al. 2003) and physiological reorganisation of grey and white matter during human puberty and adolescence. The synthesis of brain neurosteroids declines with age,

during stressful conditions, and in chronic inflammatory and neurodegenerative diseases. Lowered levels of "counter-regulatory" ALLOP and DHEA contribute to the development of diminished neurotrophic activity and accelerated cell ageing (Wolkowitz et al. 2011).

ALLOP and DHEA stimulate HPA axis activity, thus inducing corticotropin-releasing hormone and/or arginine vasopressin synthesis. These neurosteroids induce rapid effects, likely via neurotransmitter receptors and the delayed effects perhaps after being metabolised to other neuroactive steroids. In addition to the stimulation of the HPA axis, some neurosteroids have antidepressant effects (Evans et al. 2012), which could be mediated by brain-derived neurotrophic factor (BDNF) and could partially explain the trophic properties of these molecules (Naert et al. 2007). These trophic effects (Nakao et al. 2011) might be mediated in part by changes in the levels of the neurosteroids observed in this study. A meta-analysis of voxel-based morphometry studies in children and adults with ADHD suggests that the use of stimulant medication may help patients progressively attenuate their developmental delay as they get older and may be associated with the normalisation of structural abnormalities in ADHD (Hart et al. 2013).

Regarding the daily fluctuation in both neurosteroid values that we analysed, there was an absence of daily changes for ALLOP and significant day/night alterations, with higher DHEA morning values, which were more significant after treatment. Such changes implicate DHEA in the physiology of the circadian rhythm related to alertness/activity to greater extent than ALLOP.

Our study, although with an open design and lack of randomization, reports objective neuroendocrine measures of response after chronic treatment. Other limitations includes the following: (1) a smaller control group that is largely composed of the siblings of the patients, because a control group exclusively consisting of unrelated healthy children might have resulted in even higher clinical and neurosteroid differences than those we report; (2) a low number of females, adolescents and patients belonging to the PAD subtype; and (3) a large proportion of ADHD children with co-morbid CD. Similar studies involving homogeneous groups of patients in terms of age, gender and co-morbidities, together with the quantification of different neurosteroids or other neuroendocrine mediators, along with a more precise estimation of the adherence to treatment, might contribute to defining biomarkers of the disorder and its co-morbidities.

In summary, our study suggests that ALLOP and DHEA may participate both in the pathogenesis of ADHD and in the therapeutic effects of methylphenidate. The differential response of ALLOP and DHEA to methylphenidate is consistent with experimental data that suggest the dissociation of effects of pharmacologic manipulations on attention and impulse control (Davies et al. 2009) and may be a disease marker for the inattentive type with respect to ALLOP and of the hyperactive type with respect to DHEA. Our results support the future clinical trials with drugs which may increase these neuroendocrine mediators with a good clinical profile.

Acknowledgments Funding for this study was provided by the Health Research Fund (FIS; Spanish Ministry of Science and Innovation), FIS-PI07-0603; the FIS had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. We wish to thank to Ms. Puri Ubago-Corpas for her entire supervision and support.

Conflict of interest All of the researchers declare that they have no conflicts of interest.

References

American Psychiatric Association (2002) Manual diagnóstico y estadístico de los trastornos mentales (DSM-IV-TR). Masson, Barcelona

Amin Z, Mason GF, Cavus I, Krystal JH, Rothman DL, Epperson CN (2006) The interaction of neuroactive steroids and GABA in the development of neuropsychiatric disorders in women. Pharmacol Biochem Behav 84:635–643

Andreen L, Nyberg S, Turkmen S, van Wingen G, Fernandez G, Backstrom T (2009) Sex steroid induced negative mood may be explained by the paradoxical effect mediated by GABAA modulators. Psychoneuroendocrinology 34:1121–1132

Barkley RA (1997) Behavioral inhibition, sustained attention, and executive functions: constructing a unifying theory of ADHD. Psychol Bull 121:65–94

Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA, Almeida OF, Sousa N (2009) The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. Mol Psychiatry 14(764–73):739

Biederman J, Faraone SV, Petty C, Martelon M, Woodworth KY, Wozniak J (2013) Further evidence that pediatric-onset bipolar disorder comorbid with ADHD represents a distinct subtype: results from a large controlled family study. J Psychiat Res 47:15–22

Brickenkamp R (1997) D2, Test de Atención. Adaptación Española. TEA ediciones, Madrid

Brookes KJ, Hawi Z, Kirley A, Barry E, Gill M, Kent L (2008) Association of the steroid sulfatase (STS) gene with attention deficit hyperactivity disorder. Am J Med Genet B Neuropsychiatr Genet 147B:1531–1535

Charalampopoulos I, Dermitzaki E, Vardouli L, Tsatsanis C, Stournaras C, Margioris AN, Gravanis A (2005) Dehydroepiandrosterone sulfate and allopregnanolone directly stimulate catecholamine production via induction of tyrosine hydroxylase and secretion by affecting actin polymerization. Endocrinology 146:3309–3318

Charalampopoulos I, Remboutsika E, Margioris AN, Gravanis A (2008) Neurosteroids as modulators of neurogenesis and neuronal survival. Trends Endocrinol Metab 19:300–307

Compagnone NA, Mellon SH (2000) Neurosteroids: biosynthesis and function of these novel neuromodulators. Front Neuroendocrinol 21:1–56

Connor DF, Ford JD (2012) Comorbid symptom severity in attention-deficit/hyperactivity disorder: a clinical study. J Clin Psychiatry 73: 711–717

Davies W, Humby T, Kong W, Otter T, Burgoyne PS, Wilkinson LS (2009) Converging pharmacological and genetic evidence indicates a role for steroid sulfatase in attention. Biol Psychiatry 66:360–367

Evans J, Sun Y, McGregor A, Connor B (2012) Allopregnanolone regulates neurogenesis and depressive/anxiety-like behaviour in a social isolation rodent model of chronic stress. Neuropharmacology 63:1315–1326

Fadalti M, Petraglia F, Luisi S, Bernardi F, Casarosa E, Ferrari E, Luisi M, Saggese G, Genazzani AR, Bernasconi S (1999) Changes of serum allopregnanolone levels in the first 2 years of life and during pubertal development. Pediatr Res 46:323–327

Farré-Riba A, Narbona J (1997) Escalas de Conners en la evaluación del trastorno por déficit de atención con hiperactividad: nuevo estudio factorial en niños españoles. Rev Neurol 25:200–204

Galderisi S, Mucci A, Monteleone P, Sorrentino D, Piegari G, Maj M (2003) Neurocognitive functioning in subjects with eating disorders: the influence of neuroactive steroids. Biol Psychiatry 53:921–927

George O, Vallee M, Vitiello S, Le MM, Piazza PV, Mayo W (2010) Low brain allopregnanolone levels mediate flattened circadian activity associated with memory impairments in aged rats. Biol Psychiatry 68:956–963

Girdler SS, Klatzkin R (2007) Neurosteroids in the context of stress: implications for depressive disorders. Pharmacol Ther 116:125–139

Grosso S, Luisi S, Mostardini R, Matera M, Barlocco EG, Casarosa E, Balestri P, Petraglia F (2011) Circulating levels of allopregnanolone, a neuroactive steroid, and leptin during treatment with valproic acid in children with epilepsy. Neuroendocrinology 93:159–164

Gupta R, Kar BR (2009) Development of attentional processes in ADHD and normal children. Prog Brain Res 176:259–276

Hart H, Radua J, Nakao T, Mataix-Cols D, Rubia K (2013) Meta-analysis of functional magnetic resonance imaging studies of inhibition and attention in attention-deficit/hyperactivity disorder: exploring task-specific, stimulant medication, and age effects. JAMA Psychiatry 70:185–198

Jagasia R, Steib K, Englberger E, Herold S, Faus-Kessler T, Saxe M, Gage FH, Song H, Lie DC (2009) GABA-cAMP response element-binding protein signaling regulates maturation and survival of newly generated neurons in the adult hippocampus. J Neurosci 29:7966–7977

Johansson AG, Nikamo P, Schalling M, Landen M (2011) AKR1C4 gene variant associated with low euthymic serum progesterone and a history of mood irritability in males with bipolar disorder. J Affect Disord 133:346–351

Kaufman AS, Kaufman ND (1996) KBIT. Test breve de inteligencia de Kaufmann. TEA ediciones, Madrid

Kovacs M (1992) Manual for the children's depression inventory. Multi-Health Systems, North Tonawanda, NJ

Kowatch RA, Youngstrom EA, Danielyan A, Findling RL (2005) Review and meta-analysis of the phenomenology and clinical characteristics of mania in children and adolescents. Bipolar Disord 7:483–496

Kretschmer BD (1999) Modulation of the mesolimbic dopamine system by glutamate: role of NMDA receptors. J Neurochem 73:839–848

Maayan R, Yoran-Hegesh R, Strous R, Nechmad A, Averbuch E, Weizman A, Spivak B (2003) Three-month treatment course of methylphenidate increases plasma levels of dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulfate (DHEA-S) in attention deficit hyperactivity disorder. Neuropsychobiology 48:111–115

Matthews M, Nigg JT, Fair DA (2013) Attention deficit hyperactivity disorder. Curr Top Behav Neurosci 1–32. doi:10.1007/7854_2013_249

Miller MN, Miller BE (2001) Premenstrual exacerbations of mood disorders. Psychopharmacol Bull 35:135–149

Moghaddam B, Roth RH, Bunney BS (1990) Characterization of dopamine release in the rat medial prefrontal cortex as assessed by *in vivo* microdialysis: comparison to the striatum. Neuroscience 36:669–676

Molina-Carballo A, Muñoz-Hoyos A, Sánchez-Forte M, Uberos-Fernández J, Moreno-Madrid F, Acuña-Castroviejo D (2007) Melatonin increases following convulsive seizures may be related to its anticonvulsant properties at physiological concentrations. Neuropediatrics 38:122–125

Molina-Carballo A, Naranjo-Gómez A, Uberos J, Justicia-Martínez F, Ruiz-Ramos MJ, Cubero-Millán MI, Contreras-Chova F, Augustin-Morales MC, Khaldy-Belkadi H, Muñoz-Hoyos A (2013) Methylphenidate effects on blood serotonin and melatonin levels may help to synchronise biological rhythms in children with ADHD. J Psychiat Res 47:377–383

Muñoz-Hoyos A, Molina-Carballo A, Augustin-Morales MC, Contreras-Chova F, Naranjo-Gómez A, Justicia-Martínez F, Uberos-Fernández J (2011) Psychological dwarfism: psychopathological and putative neuroendocrine markers. Psychiat Res 188:96–101

Naert G, Maurice T, Tapia-Arancibia L, Givalois L (2007) Neuroactive steroids modulate HPA axis activity and cerebral brain-derived neurotrophic factor (BDNF) protein levels in adult male rats. Psychoneuroendocrinology 32:1062–1078

Nakao T, Radua J, Rubia K, Mataix-Cols D (2011) Gray matter volume abnormalities in ADHD: voxel-based meta-analysis exploring the effects of age and stimulant medication. Am J Psychiatry 168:1154–1163

Nigg JT (2005) Neuropsychologic theory and findings in attention-deficit/hyperactivity disorder: the state of the field and salient challenges for the coming decade. Biol Psychiatry 57:1424–1435

Nigg JT, Casey BJ (2005) An integrative theory of attention-deficit/hyperactivity disorder based on the cognitive and affective neurosciences. Dev Psychopathol 17:785–806

Pinna G, Costa E, Guidotti A (2005) Changes in brain testosterone and allopregnanolone biosynthesis elicit aggressive behavior. Proc Natl Acad Sci U S A 102:2135–2140

Pluchino N, Cubeddu A, Giannini A, Merlini S, Cela V, Angioni S, Genazzani AR (2009) Progestogens and brain: an update. Maturitas 62:349–355

Predieri B, Luisi S, Casarosa E, De SM, Balli F, Bernasconi S, Rossi M, Petraglia F, Iughetti L (2007) High basal serum allopregnanolone levels in overweight girls. Int J Obes (Lond) 31:543–549

Puia G, Gullo F, Dossi E, Lecchi M, Wanke E (2012) Novel modulatory effects of neurosteroids and benzodiazepines on excitatory and inhibitory neurons excitability: a multi-electrode array recording study. Front Neural Circuits 6:94

Sánchez CR, Ramos C, Díaz F, Simón M (2010) Validación de la escala de evaluación del trastorno por déficit de atención/hiperactividad (EDAH) en población adolescente. Rev Neurol 50:283–290

Strous RD, Spivak B, Yoran-Hegesh R, Maayan R, Averbuch E, Kotler M, Mester R, Weizman A (2001) Analysis of neurosteroid levels in attention deficit hyperactivity disorder. Int J Neuropsychopharmacol 4:259–264

Traish AM, Kang HP, Saad F, Guay AT (2011) Dehydroepiandrosterone (DHEA)—a precursor steroid or an active hormone in human physiology. J Sex Med 8:2960–2982

Trent S, Dean R, Veit B, Cassano T, Bedse G, Ojarikre OA, Humby T, Davies W (2013) Biological mechanisms associated with increased perseveration and hyperactivity in a genetic mouse model of neurodevelopmental disorder. Psychoneuroendocrinology 38:1370–1380

van Ewijk H, Heslenfeld DJ, Zwiers MP, Buitelaar JK, Oosterlaan J (2012) Diffusion tensor imaging in attention deficit/hyperactivity disorder: a systematic review and meta-analysis. Neurosci Biobehav Rev 36:1093–1106

van Goozen SH, van den Ban E, Matthys W, Cohen-Kettenis PT, Thijssen JH, van Engeland H (2000) Increased adrenal androgen functioning in children with oppositional defiant disorder: a comparison with psychiatric and normal controls. J Am Acad Child Adolesc Psychiatry 39:1446–1451

Wang LJ, Hsiao CC, Huang YS, Chiang YL, Ree SC, Chen YC, Wu YW, Wu CC, Shang ZY, Chen CK (2011) Association of salivary dehydroepiandrosterone levels and symptoms in patients with attention

deficit hyperactivity disorder during six months of treatment with methylphenidate. Psychoneuroendocrinology 36:1209–1216

Wiltschko AB, Pettibone JR, Berke JD (2010) Opposite effects of stimulant and antipsychotic drugs on striatal fast-spiking interneurons. Neuropsychopharmacology 35:1261–1270

Wolkowitz OM, Reus VI, Mellon SH (2011) Of sound mind and body: depression, disease, and accelerated aging. Dialogues Clin Neurosci 13:25–39

Yang SY, He XY, Olpin SE, Sutton VR, McMenamin J, Philipp M, Denman RB, Malik M (2009) Mental retardation linked to mutations in the HSD17B10 gene interfering with neurosteroid and isoleucine metabolism. Proc Natl Acad Sci U S A 106:14820–14824

Zambrano-Sánchez E, Martínez-Cortés JA, Del Río-Carlos Y, Martinez-Wbaldo MD, Poblano A (2011) Identification of attention-deficit-hyperactivity disorder and conduct disorder in Mexican children by the scale of evaluation of deficit of attention and hyperactivity scale. Psychiatry Res 187:437–440

Zheng P (2009) Neuroactive steroid regulation of neurotransmitter release in the CNS: action, mechanism and possible significance. Prog Neurobiol 89:134–152