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Prepubertal gonadotropin-releasing hormone analog leads to exaggerated behavioral and emotional sex differences in sheep

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ABSTRACT

In mammals, sex specialization is reflected by differences in brain anatomy and function. Measurable differences are documented in reproductive behavior, cognition, and emotion. We hypothesized that gonadotropin-releasing hormone (GnRH) plays a crucial role in controlling the extent of the brain's sex specificity and that changes in GnRH action during critical periods of brain development, such as puberty, will result in altered sex-specific behavioral and physiological patterns. We blocked puberty in half of the 48 same-sex Scottish mule Texel cross sheep twins with GnRH analog (GnRHa) goserelin acetate every 3 weeks, beginning just before puberty. To determine the effects of GnRHa treatment on sex-specific behavior and emotion regulation in different social contexts, we employed the food acquisition task (FAT) and measurement of heart rate variability (HRV). ANOVA revealed significant sex and sex × treatment interaction effects, suggesting that treated males were more likely to leave their companions to acquire food than untreated, while the opposite effect was observed in females. Concordant results were seen in HRV; treated males displayed higher HRV than untreated, while the reverse pattern was found in females, as shown by significant sex and sex × treatment interaction effects. We conclude that long-term prepubertal GnRHa treatment significantly affected sex-specific brain development, which impacted emotion and behavior regulation in sheep. These results suggest that GnRH is a modulator of cognitive function in the developing brain and that the sexes are differentially affected by GnRH modulation.

Introduction

Male and female specialization is reflected by distinct brain anatomy and function (Baum, 2006). In humans, measurable differences exist, not only in reproductive behavior but also in cognitive functions. These include language, visuospatial information processing, and memory (Andreano and Cahill, 2009). Sex-specific differences, however, are quite selective in terms of the spectrum of cognitive brain functions as a whole, and their magnitude vary during the life course. Much remains to be discovered as to how the balance between sex-specific specialization and non-sex-specific functioning is implemented and how imbalance affects brain diseases. Recently, research has focused on the effects of gonadotropin-releasing hormone (GnRH) and its synthetic analogs (GnRHa) on sex-specific cognitive and physiological patterns, initiated by the fact that GnRH receptor expression has been found in various brain areas and peripheral tissues unrelated to reproduction (Skinner et al., 2009; Wilson et al., 2006). Results in adult human males and females, as well as in rodents, indicate that GnRHa may lead to significant changes in several cognitive functions. Subtle but significant impairments in domains of visuospatial and higher-order executive control functions (Nelson et al., 2008), as well as episodic increase of depressive symptoms (Schmidt et al., 2004), were discovered in men. In women, GnRHa were associated with decline in working memory (Grigorova et al., 2006; Palomba et al., 2004) and with disrupted encoding in episodic verbal memory (Craig et al., 2007). On the other hand, in animal models of Alzheimer's disease, GnRHa had positive effects on

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cognitive function (Bryan et al., 2009). Unfortunately, this research is done only in adults. Comparable studies in children and adolescents are lacking (Carel, 2005), although biological mechanisms of sex-specific brain development during puberty are not sufficiently understood (Jazin and Cahill, 2010). This general lack of knowledge of GnRHa effects on cognitive and behavioral development is reflected in the recently published "Consensus Statement on the Use of Gonadotropin-Releasing Hormone Analogs in Children" (Carel et al., 2009). Nevertheless, GnRHa are widely used in treatment of various conditions in children, such as precocious puberty.

Cognitive functioning and, in particular, higher-order control functions improve gradually from childhood to adulthood. Paradoxically, at the same time, puberty is associated with increased psychological vulnerability. During this period, overall morbidity and mortality increase by 200%, which is mostly related to risk-taking behavior and emotional problems (Dahl, 2004; Eaton et al., 2008). Typically, males tend to engage in more novelty seeking and risk-taking while females display more fear and avoidance behavior (Kerschbaum et al., 2006). Similar sex differences are found in sheep (Boissy et al., 2005). The question arises as to whether manipulation of GnRH may affect these developmental processes. We propose in this paper that (a) GnRH plays a crucial role in controlling the extent of the brain's sex specificity, and (b) changes in the availability of this decapeptide during critical periods of brain development (such as at puberty) are reflected in altered sex-specific behavioral and physiological patterns.

To our knowledge, this is the first study to investigate the sex-specific cognitive development effects of prepubertal GnRHa treatment in mammals. In this paper, we present the results of experiments exploring emotion and behavior regulation. These functions develop and change rapidly during puberty and have been associated with increased vulnerability (Casey et al., 2008). Regulation can be defined as the ability to flexibly control emotions and behaviors according to the demands of the current situation. This has been closely linked to a peripheral physiological parameter, heart rate variability (HRV). In humans, higher resting HRV is associated with enhanced capacity to control emotions, thoughts, and behaviors (Appelhans and Luecken, 2006). Similarly, in sheep, presumed negative and positive emotional states were shown to differ with respect to HRV (Reefmann et al., 2009).The central autonomic network (CAN), consisting of prefrontal and limbic brain structures, is postulated as the functional unit through which the brain controls cognitive, behavioral, and physiological responses to emotional states. This is accomplished by inhibiting maladaptive and context-irrelevant responses via the vagus nerve in the periphery. This neurovisceral integration relates emotion regulation to HRV in a dynamic systems framework (Thayer and Lane, 2009). Therefore, HRV is considered to be an index of the CAN's ability to regulate the emotional response through inhibition so that it is appropriate to the current situation. Low HRV has been associated with hypervigilance and a defensive behavioral system, while high HRV has been associated with a flexible emotion-modulated response (Ruiz-Padial et al., 2003).

We chose to employ sheep to investigate the effects of prepubertal GnRHa treatment on aspects of emotion and behavior regulation because of their long period of brain development compared to other mammals, such as rodents. We used a behavioral experiment, i.e., a food acquisition task (FAT), to assess the animals' ability to engage in reward-seeking behavior while inhibiting the negative emotions provoked by separation from their companions. In addition, we assessed CAN activity by measuring HRV in each animal under unrestrained, socially neutral conditions.

Materials and methods

Subjects

The experiment was conducted at the University of Glasgow's Cochno Research Centre using 48 pairs of same-sex Scottish mule Texel cross twin lambs. All animal procedures were conducted in accordance with home office regulations (pil 60/3826). Same-sex twins were included in the study to eliminate the possible developmental effects of steroid transfer between siblings of different sexes. The lambs remained with their dams until weaning and were maintained under standard husbandry conditions. Within each set of twins, one was randomly assigned to the control (C) and the other, to the treatment (T) group. Males and females were separated during the entire study. Animals in the T group received the GnRHa goserelin acetate (Zoladex, AstraZeneca; 3.75 mg every 3 weeks) beginning just prior to the time of puberty at 10 weeks of age in males and 30 weeks of age in females because of the sex-specific timing of puberty in this species (Wood and Foster, 1998). Blood serum analyses were performed regularly during the animals' life, and after their death, at 12 months of age, the heart and testes or ovaries were excised, weighed, and histologically evaluated (Table 1). The analyses confirmed that treatment prevented puberty by complete suppression of the hypothalamus–pituitary–gonadal axis.

Food acquisition task

The FAT took place when the animals were 1 year old. They were divided into groups of twelve sheep each. Each animal was trained and tested within its group.

Training phase

Each test-group spent a night in the test arena (Fig. 1) moving freely, with hay and water available *ad libitum*. On the following morning, in addition to hay in the "hay arena" (HA) side, presumably highly attractive pellets were placed in the "pellet arena" (PA) side. Each test-group was led through the test arena, starting at the HA gate and moving at their own pace to the PA side. It was assured that every animal tasted pellets. Thereafter, every sheep made 18 individual training-runs according to the same procedure as under group training, while its 11 companions waited in the audience pen. After each run, an animal was reunited with its peers and another one underwent the same procedure. During the last 8 runs of individual training, by randomization, pellets were provided at the 4th and 8th runs only. This corresponded to the pellet occurrence in the actual test.

Test phase

Testing started between 1600 and 1630 h, 2 h after the end of individual training and consisted of eight 1 min runs. After each single run, an animal was reunited with the rest of the test-group in the audience pen. Pellets (7 ml) were silently poured into the feeder at the 4th and 8th runs, providing an intermittent reward schedule. At the start of each run, an animal was placed at the HA gate while its 11 companions were in the audience pen, approximately 1.5 m away. Human observers were hidden. The test-run was deemed complete when the animal either ate hay in the HA side or tried to obtain pellets

Table 1
Body and organ weights at 12 months of age.

Sex	Group	Body [kg] mean ± SD (*n*)	Testes [g]* mean ± SD (*n*)	Ovaries [g]* mean ± SD (*n*)	Heart [g] mean ± SD (*n*)
Male	C	44.50 ± 1.30 (21)	76.01 ± 3.67 (21)	–	197.50 ± 6.69 (21)
	T	44.17 ± 0.91 (21)	55.55 ± 4.49 (20)	–	200.00 ± 4.17 (20)
Female	C	35.05 ± 1.08 (20)	–	0.69 ± 0.04 (20)	183.54 ± 7.61 (20)
	T	35.40 ± 0.89 (20)	–	0.50 ± 0.04 (20)	175.90 ± 4.30 (20)

C indicates control group; T, treatment group.
* Testes and ovaries were significantly smaller in T vs. C groups ($p<0.01$).

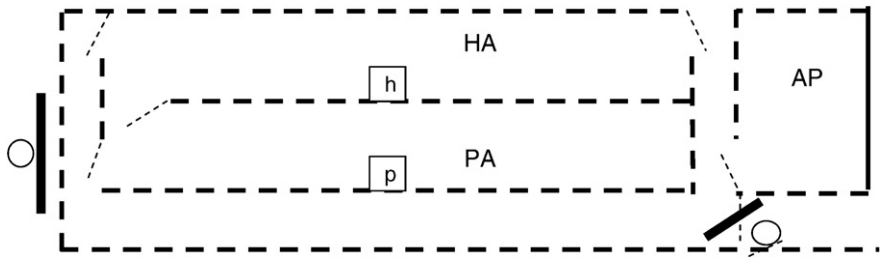

Fig. 1. Food acquisition task experimental setup. AP indicates audience pen; HA, hay arena; PA, pellets arena; h, containers with hay; p, containers with pellets. Thick stippled line, 1 m high barred barrier; thin stippled line, gates; solid line, plastic screen with a spy-hole; circle, human observer.

from the PA feeder. If the animal did neither within the test period, it was calmly ushered out of the arena through a third, remote gate and was reunited with its companions in the audience pen.

Scoring

After each run, it was recorded whether an animal moved farther away from its companions in the audience pen to acquire the food and, if so, whether it went after the hay or pellets. The FAT score was calculated as a proportion of how many times, out of 8 test runs, a sheep chose to move farther away from its companions to get the food, regardless of the type of it. To determine whether food acquisition behavior was mainly motivated by hay or by pellets, correlations between FAT scores and number of "hay runs" (test animal ate hay) and "pellet runs" (test animal tried to obtain pellets) were calculated.

Heart rate variability

HRV was measured twice, at 7 (T1) and 12 (T2) months of age. At T1, males in the T group had already been treated for 20 weeks, whereas females in the T group were about to start their treatment. Heart action was recorded as inter-beat intervals (IBI) using a Polar sports tester (Polar RS800). To ensure sufficient contact between the detector and skin, animals were shaved on the ventral region of the chest the evening before testing. After attaching the detector to the elastic strap, the animals were returned to their social groups in their home pen, and recordings were collected for 10 min. The last 5 min of the recordings were used in the analyses after being scrutinized for artifacts, according to the algorithm described by Berntson et al. (1990). The identified measurement errors were substituted by interpolations of neighboring IBI. If the number of artifacts exceeded 5% of total IBI, the data set was excluded from analysis. Statistical HRV parameters in the time and frequency domains were computed according to the HRV Task Force recommendations (1996). High-frequency (HF) measure (0.04–0.15 Hz) was chosen as the main parameter for estimation of cardiac vagal control.

Statistical analyses

Two-way between-groups analysis of variance (ANOVA) was used to calculate the differences in FAT scores between T and C groups, sexes, and interaction effects between sex and treatment. In addition, a mixed between–within subjects ANOVA was conducted to assess the impact of the treatment on FAT scores in twin pairs.

HRV time domain and frequency spectrum measures were used to check the consistency between various HRV parameters. HF was logarithmically transformed (HF(ln)) to normalize the data. Differences between HF(ln) at T1 and T2 were calculated using paired sample *t*-tests to establish the development in cardiac vagal control over time in male and female T and C groups. The analyses of HRV differences were done on the basis of HF(ln) values at T2. Two-by-two between-groups analysis of covariance (ANCOVA), with body and heart weight as covariates, was used to estimate the differences between T and C groups, sexes, and interaction effects between sex and treatment. Because all same-sex twin pairs were split into C and T groups, additional statistics were performed to establish the mean difference in HF(ln) between twins.

In post hoc analyses, effect sizes for mean differences between T and C groups for males and females were calculated by means of Cohen's *d* for both FAT and HF(ln).

Results

Sex differences

Out of initially 96 sheep, 8 animals dropped out because of bad health or death. As expected, in control groups, we observed only slight and insignificant sex differences in FAT scores and HF(ln); males had slightly higher FAT score and HF(ln) than females. In the treatment groups, these sex differences became greatly exaggerated and highly significant (Fig. 2).

Food acquisition task

ANOVA revealed a significant sex effect ($F(1, 84) = 10.19$, $p = 0.002$) and a significant interaction between sex and treatment ($F(1, 84) = 4.2$, $p = 0.04$), indicating that treated males were more likely to move away from their companions to acquire food than untreated males, while the opposite effect was observed in females (Fig. 2A). The effect size of the differences between males in the T and C groups ($d = 0.60$) was larger and opposite from what was observed in females ($d = -0.28$). To control for the effects of potential genetic factors on behavior, the sample size of 22 male twin pairs and 20 female pairs was analyzed with mixed between–within subjects ANOVA. The results showed a significant sex effect ($F(1, 40) = 6.11$, $p = 0.018$), whereas the interaction between sex and treatment approached significance ($F(1, 40) = 3.17$, $p = 0.083$).

The impact of "safe hay" and "risky pellets" on FAT score

To determine whether the animals stayed at the first-available food source (hay) or continued to walk to the intermittently available pellets, we correlated FAT scores with the number of "hay runs" and "pellet runs". Controls and treated males, as well as female controls, were highly motivated by "risky pellets" in their search for food. Spearman's *R* values between FAT and "pellet runs" were 0.8–0.9 ($p < 0.005$) in these groups. Treated females, however, were mostly attracted to "safe hay," as reflected by a high correlation between FAT scores and "hay runs" ($R = 0.9$, $p < 0.005$).

Heart rate variability

Thirty-one animals were excluded from the analyses due to conservative evaluation of recording artifacts. A two-by-two between-groups ANCOVA was conducted to assess the impact of treatment on HF (ln) in males and females at T2. Body and heart weight were used as

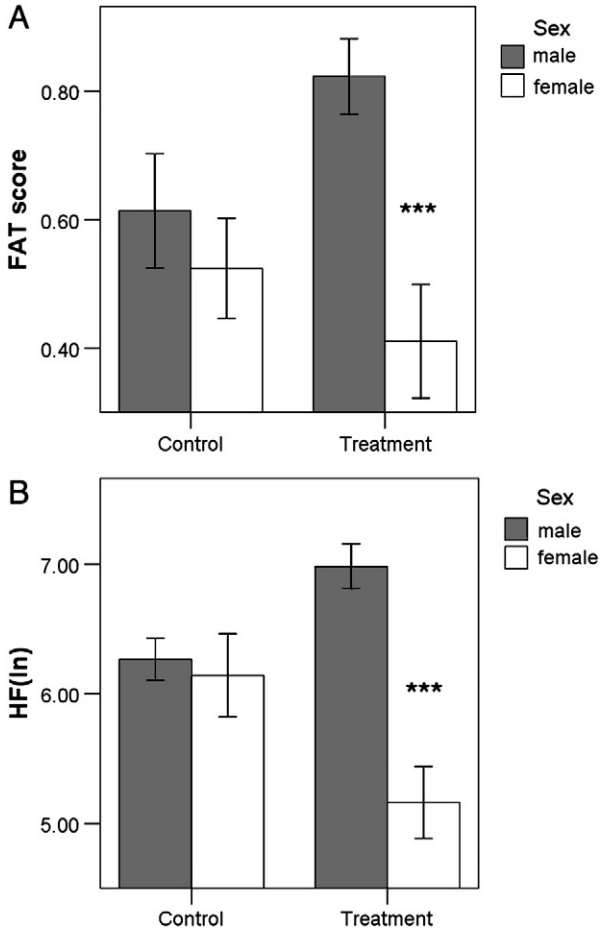

Fig. 2. Treatment effects of puberty blockage with gonadotropin-releasing hormone analog on (A) food acquisition task, and (B) heart rate variability in male and female lambs at 12 months of age (mean ± standard error of the mean). FAT indicates food acquisition task; HF(ln), logarithmically transformed high-frequency of heart rate variability. ***$p<0.0005$.

covariates in the model. Treated males showed higher HF(ln) than untreated animals, suggesting higher vagally mediated cardiac control, while the opposite findings were observed in females (Fig. 2B). ANCOVA showed a significant effect of sex ($F(1, 49) = 14.33$, $p<0.0005$) and interaction effect between sex and treatment in HF(ln) ($F(1, 49) = 10.93$, $p = 0.002$). The effect size of differences between males in the T and C groups ($d = 1.01$) was of similar size but in opposite directions to what was recorded in females ($d = -0.98$). To control for the effects of genetic factors on HRV, 14 male twin pairs were analyzed. The effect size of differences between male twins was approximately the same as at the group level ($d = 1.02$). Female twin pairs were not analyzed because only four pairs had complete data sets at this point. Longitudinal analyses of HRV data (T1 and T2) showed that tendencies towards differences between the groups at T1 became greater at T2.

Discussion

We found that animals with blocked puberty displayed greater sex differences in relation to emotion and behavior regulation than their untreated twins. Treated males were more willing to move away from their companions compared to male controls and treated and untreated females. They were highly motivated by the prospect of obtaining "risky pellets," as shown by high correlations between FAT scores and "pellet runs." This exaggerated "maleness" could be interpreted as an expression of improved emotional control compared to their same-sex twins. One may speculate that they were able to better inhibit negative emotions, such as anxiety. On the other hand, this changed behavior could also be interpreted as being uncritical since a typical treated male went repeatedly after pellets even though they were to be found only twice in eight runs. However, the fact that the experiment was relatively risk-free and that the animal was on its own, not competing with other males, might favor the interpretation of improved emotional control as the key factor. In contrary to treated males, treated females tended to stay in the same place, as close as possible to their companions in the audience pen, and were much less prone to engage in food seeking. If they sought food, it was more often the nearby and visible hay than the remotely placed and out-of-sight pellets. This avoidance behavior might be interpreted as poor emotional control, leading to higher anxiety at the prospect of farther separation from the other animals.

The HRV results complemented behavioral findings. We found that long-term treatment with GnRHa affected cardiac vagal control in young sheep in a sex-specific manner. Treated males had significantly higher HRV (i.e., larger cardiac vagal influence) than untreated males and females. The HRV differences between treated males and females were significantly exaggerated compared to untreated animals. Larger cardiac vagal influence is thought to be related to better emotional control and a better ability to engage in adaptive behavior in various circumstances. It was previously shown in sheep that HRV differs with relation to positive and negative emotional states (Reefmann et al., 2009). Poor emotional control, e.g., in case of anxiety, is associated with lower HRV. HRV findings seem robust, despite our conservative approach to artifact-contaminated data records, which resulted in the rejection of 31 cases. Comparison of the T1 and T2 results showed consistency in HRV findings; the tendencies present at T1 increased with time.

It can be argued that differences between sexes in treatment effect size (FAT score) might be attributed to a 20 weeks longer treatment period in males. We cannot confirm or reject this possibility on the basis of the present data. However, if this is true, our findings may be even more significant. The effect of the treatment in males was opposite to what we observed in females, leading to exaggerated sex differences. If longer treatment increases this diversity, it is even more important to urgently follow up these findings in human studies. On the other hand, our results did not suggest that treatment duration had an impact on all effect sizes. In the case of HRV, treatment effects in males and females were of similar magnitude and in the opposite directions.

A possibility of non-central nervous system (CNS)-related GnRHa effects on HRV should also be considered. In adult humans, androgen deprivation therapy (with GnRHa) is associated with increased risk of cardiovascular disease (Saigal et al., 2007), and GnRH receptors are found in peripheral tissues, including the heart (Skinner et al., 2009). However, the direct effects of receptor blockage on heart function have so far not been sufficiently documented. Even though we cannot rule out the possibility that the treatment itself might have had an influence on HRV unrelated to CNS function, androgen deprivation should lead to poorer HRV, while the current results suggest significant HRV increases, at least in treated males.

In humans, puberty is characterized by high reward sensitivity, often expressed by impulsive, "control-lacking" and risk-taking behavior, which is more pronounced in young males, while young females tend to experience more emotional problems (Eaton et al., 2008; Garber, 2006). This has been attributed to a rivalry between rapid changes in reward sensitivity systems following the development of limbic brain structures (e.g., nucleus accumbens), and relatively slower improvements in cognitive control capacity associated with the development of prefrontal brain areas involved in top-down control (Casey et al., 2008). Although the direct translation of the results from animal models to humans is questionable, the behavior observed in our animals could be a result of influence on the similar brain development related phenomena. Therefore, we

hypothesize that GnRHa treatment may impact these developmental processes in young humans as well. Confirmation or rejection of this hypothesis needs to emerge as a result of comparable human studies. At present, our group is conducting human studies, employing more advanced assessment techniques (i.e., neuropsychological tests, HRV measurements, functional magnetic resonance imaging).

Mechanisms

Our current findings raise the question about how GnRHa affects behavior and emotion regulation and, thereby, brain development. Blockage of the reproductive axis leads to decreased gonadotropin and sex steroid secretion, which has traditionally been suggested to be the main reason for cognitive changes in adults treated with GnRHa. However, our results suggest a different scenario. The sex-specific effects documented in the present study may be related to direct influence on brain areas unrelated to reproductive function. As recently shown, GnRH type 1 receptors (GnRHR 1) are widely expressed throughout the mammal brain, e.g., GnRHR 1 is localized to the cell body and apical dendrites of pyramidal neurons in the hippocampus of humans and in other species, including sheep (Skinner et al., 2009; Wilson et al., 2006). Studies on rodents have demonstrated that intracerebral injection of synthetic GnRH into the rat brain leads to changes in conditioned avoidance responses to electric foot-shock stimuli with biphasic effects: small dosages increased the ratio of successful avoidance responses while high doses decreased it (Mora et al., 1991, 1998). These findings support the possibility that GnRHa directly affects cognition and behavior. However, it may be argued that another agent, gonadotropin-inhibitory hormone (GnIH), may interact with GnRHa actions in relation to potentially stressful situations (e.g., assessment procedures). Rodent studies have shown that stress leads to increase in GnIH production which inhibits GnRH (Kirby et al., 2009). Nevertheless, since both treated and untreated animals were subjected to the same procedures, the observed differences may be treatment related. In accordance with de Vries and Sodersten (2009), one might argue that sex differences may have dimorphic effects on behavior in that the removal of existing sex differences may itself lead to exaggerated dimorphic sex-related behavior. In such a scenario, GnRHa may have blocked sex steroid influences on brain development and, therefore, may have exaggerated behavioral sex differences.

Possible implications

There is a need for personalized drugs with improved efficiency on sex-specific behavioral symptoms for many psychiatric and somatic disorders. Women suffer more often from depression, late-onset schizophrenia, and Alzheimer's disease, whereas autism is considerably more common in men (Compton et al., 2002). Sex-specific GnRH treatment options could modulate sex-specific differential cellular responses to GnRH and thereby change sex-specific behavior changes in humans during brain development and aging. We propose that it is the increased postnatal gonadotropin concentration, in puberty and in older age, rather than the increase or decrease in steroid hormones (e.g., estrogen), that is essential for either normal brain development or an increased risk of cognitive impairment and brain disease. Therefore, gonadotropin modulation could play a role in preventing and treating cognitive impairment in patients with developmental cognitive disorders.

Conclusions

We have demonstrated, to our knowledge for the first time, significant pubertal sex-specific behavioral and neurovisceral changes in mammals that were associated with prepubertal blockage of the GnRH receptor. Our findings may potentially suggest new therapeutic applications of GnRH analogs. Currently, these analogs are exclusively used as inhibitors of the reproductive hypothalamic pituitary axis and have been discussed only recently as a possible treatment in Alzheimer-type dementia (Casadesus et al., 2006). We believe that GnRH analogs should as well be investigated as cognitive modulator drugs to treat younger patients for several diseases associated with cognitive impairment (Tourette syndrome, attention deficit hyperactivity disorder, obsessive–compulsive disorder, borderline personality disorder, and bipolar disorder type II).

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