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# Amino Acid Neurotransmission and Initiation of Puberty: Evidence from Nonketotic Hyperglycinemia in a Female Infant and Gonadotropin-Releasing Hormone Secretion by Rat Hypothalamic Explants

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## ABSTRACT

The pulse frequency of hypothalamic GnRH secretion increases at the onset of puberty. In rodents and primates, this process involves facilitatory and inhibitory effects mediated through hypothalamic *N*-methyl-D-aspartic acid (NMDA) and γ-aminobutyric acid (GABA) receptors, respectively. Precocious puberty was observed in an 11-month-old girl with nonketotic hyperglycinemia. This was thought to result from the effect of high concentrations of glycine (112 μmol/L in cerebrospinal fluid; normal, 3–12) acting on NMDA receptors as a coagonist of glutamate. Regression of pubertal development during anticonvulsive treatment with GABA agonists (loreclezole and vigabatrin) suggested that the stimulatory effects of glycine could be overcome by GABA receptor-mediated inhibition. These two hypotheses were tested in the *in vitro* model of the explanted hypothalamus from infantile (15-day-old) male rats. Glycine concentrations of 1–10 μmol/L increased the pulse frequency of GnRH secretion. This acceleration was prevented by 7-chlorokynurenic acid, a glycine antagonist at the NMDA receptor complex, and by the GABA agonist loreclezole. In addition, loreclezole and vigabatrin suppressed the developmental increase in the frequency of pulsatile GnRH secretion. The observation of precocious puberty in an infant with hyperglycinemia followed by pubertal regression during GABA agonist therapy and the *in vitro* findings in hypothalamic explants suggest that stimulatory inputs mediated through NMDA receptors and inhibitory inputs through GABA receptors are involved in the initiation of puberty.

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In many species, including man, the hypothalamus has the capacity to secrete GnRH in a pulsatile manner. The onset of puberty is preceded by an increase in the pulse frequency of GnRH secretion, as shown in portal blood of female monkeys (1) or in the incubation medium of hypothalamic explants of male rats (2, 3). In children and adolescents, a similar process is evidenced indirectly through sequential LH measurements in peripheral blood (4, 5).

The gonadotropic axis is known to be active in the human fetus and infant before the long quiescent period preceding onset of puberty (6). Precocious puberty is observed after central nervous system (CNS) irradiation (7, 8) and following experimental disconnection or lesion of particular hypothalamic areas (9, 10). These data suggest that a hypothalamic facilitatory mechanism is operational prepubertally, whereas an inhibitory or restraining mechanism is superimposed. The facilitatory mechanism may involve a subtype of glutamate receptors that selectively bind the agonist *N*-methyl-D-aspartate (NMDA), because early onset of puberty is caused by chronic intermittent administration of NMDA to infantile female rats (11) or juvenile male monkeys (12). Using pharmacological and antisense strategies *in vitro*, we showed recently that in the infantile rat hypothalamus, the drive of pulsatile GnRH secretion via NMDA receptors was restrained by a γ-aminobutyric acid$_A$ ($GABA_A$) receptor-mediated inhibition (13). This GABA-ergic inhibition showed a marked reduction at the onset of puberty (14), confirming data obtained previously in the juvenile female monkey (15).

In man, no evidence of NMDA receptor involvement in the neuroendocrine regulation of gonadotropin secretion has been provided to date. Scarce data on the inhibition of gonadotropin secretion by antiepileptic drugs such as valproic acid, a GABA agonist (16), suggest a possible inhibitory role of GABA receptors (17–19). In this preliminary report, precocious puberty in a female infant with nonketotic hyperglycinemia (NKH) was postulated to be related to glycine-mediated NMDA receptor stimulation. In addition, regression of pubertal development was observed during therapy with antiepileptic GABA agonists and was proposed to be related to GABA receptor-mediated inhibition. These hypotheses were tested *in vitro* using a rat hypothalamic explant paradigm.

# Subjects and Methods

## CASE REPORT

This girl was born at term of a normal pregnancy as the first child of healthy unrelated parents. On the day of birth, severe hypotonia was apparent. Spontaneous movements were sparse, but reactions were excessive upon stimulation. On day 5, generalized seizures were treated with phenobarbital (3–5 mg/kg·day) and phenytoin (5–10 mg/kg·day). On day 10, NKH was diagnosed based on elevated glycine concentrations in plasma (1044 μmol/L; normal range, 80–340) and cerebrospinal fluid (112 μmol/L; normal, 3–12). The infant gradually developed severe lethargy, psychomotor retardation, and convulsions refractory to conventional antiepileptic therapy.

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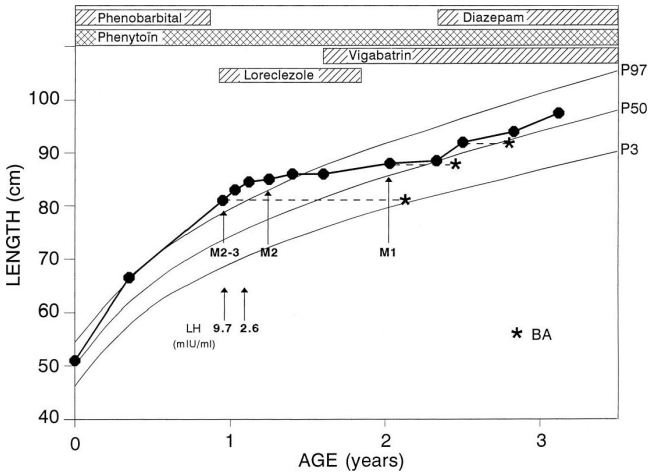
By 11 months of age, breast development was noticed and scored as stage $M_2$–$M_3$ according to Tanner (20). Height (81.4 cm) was above the 97th percentile for chronological age, and height velocity was rapid, as shown by the growth curve (**Fig. 1**). Head circumference was 45.5 cm (50th percentile). Bone age was advanced to 26 months according to the criteria of Tanner and Whitehouse (21). At 12 months, plasma LH was increased for age (9.7 mIU/mL; normal, < 2). FSH was slightly elevated (3.4 mIU/mL; normal, < 3). IGF-I was at the upper normal limit (112 μg/L; normal, 44–110). GH measured on two occasions (3.0 and 1.0 μg/L) as well as PRL (259 μU/mL) were not elevated. Sexual precocity of central origin was diagnosed, although no GnRH test was performed.

**Fig. 1.** Length in relation to age and bone age (BA) in a girl with nonketotic hyperglycinemia. The Tanner stages of breast development (M) were estimated on three occasions, and basal plasma LH levels were determined on two occasions. The *bars* show the timing of administration of various antiepileptic drugs.




The multiple daily seizure episodes did not respond to phenobarbital, which was discontinued at 11 months. As only partial improvement was seen using phenytoin, treatment with loreclezole (1 mg/kg·day) was initiated. This new antiepileptic agent (Janssen Pharmaceutica, Beerse, Belgium) has a stimulatory action at a modulatory site distinct from the benzodiazepine site within the GABA-receptor complex (22). At 15 months of age, 4 months after starting loreclezole treatment, regression of breast development ($M_2$) was noticed, and height velocity started to decrease (**Fig. 1**). Basal plasma LH was 2.6 mIU/mL. Loreclezole therapy only slightly decreased the frequency of seizure episodes and was discontinued at 22 months of age. Vigabatrin (Sabril, Marion Merrell Dow, Strasbourg, France; 30–70 mg/kg·day), another GABA agonist acting through irreversible inhibition of GABA transaminase (23), was given from 19 months of age and had a significant anticonvulsive effect. At 24 months, further evidence of reduced growth velocity was obtained, breast development disappeared ($M_1$), and bone age advancement decreased to 5 months compared to chronological age. After 27 months of age, a normal growth rate was observed (**Fig. 1**). Treatment using diazepam (1.5–3 mg/kg·day) was started. At 4 yr of age, a computed tomographic scan of the brain visualized diffuse cortical and subcortical atrophy without any focal abnormality, particularly in the hypothalamopituitary area.

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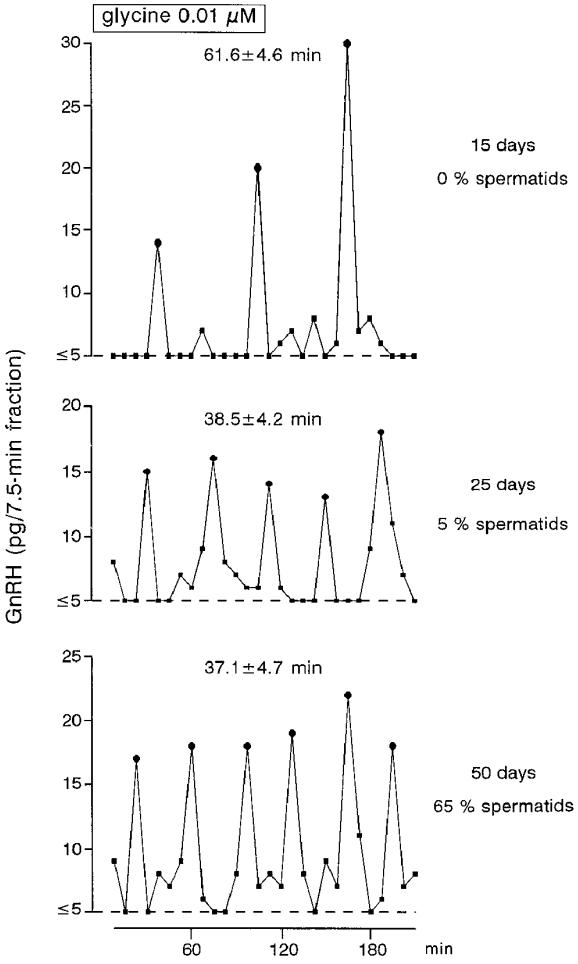
## IN VITRO STUDIES

Retrochiasmatic hypothalamic explants from 15-, 25-, or 50-day-old male rats were studied individually (12–15 explants/experiment) using a static incubation system (**Fig. 2**). The incubation medium (0.5 mL) was renewed every 7.5 min for 3–5 h, and the collected fractions were kept frozen until assayed for GnRH using a highly sensitive RIA. This procedure has been described in detail previously (5, 6). It is of note that the studied explants did not include the preoptic area where the perikarya of GnRH neurons are located in the rat brain. We confirmed recently that most of the studied explants contained no GnRH cell bodies, in contrast to the 200–400 perikarya visualized in the preoptic area (24).

**Fig. 2.** Representative profile of pulsatile GnRH secretion by individual explants of the retrochiasmatic hypothalamus from 15-, 25-, and 50-day-old male rats. The mean ± SD interpulse interval is given. The *broken lines* indicate the limit of detection of GnRH release. ●, Significant pulse (Pulsar program).




Although the incubation medium contained 400 μmol/L glycine in our early experiments (2), a reduced concentration of 0.01 μmol/L was used subsequently because it resulted in optimal GnRH release in response to NMDA (25). The developmental changes in the frequency of pulsatile GnRH secretion have been characterized in medium containing 0.01 μmol/L glycine and correlated with

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the occurrence of spermatids measured through flow cytometry in testicular homogenates, as described previously (3). Here, using explants of 15-day-old rats, we studied the effect of glycine concentration (0.0001–100 µmol/L) on the frequency of pulsatile GnRH secretion. These experiments were repeated using 7-chlorokynurenate (7CK), a specific antagonist of glycine binding at the NMDA-receptor complex (26), and strychnine, an antagonist at the non-NMDA glycine receptor in the CNS (27). Also, the effects of loreclezole and vigabatrin, two GABA agonists, were studied at 15 and 25 days. The significance of GnRH secretory pulses was determined using the Pulsar program (28), as described previously (3). The significance of changes in mean GnRH pulse frequency was calculated through ANOVA with correction for repeated measurements and by Scheffe's F test (29).

# Results

Using explants incubated with 0.01 µmol/L glycine, the frequency of pulsatile GnRH secretion was similar at 25 days, when spermatids started to occur, to that at 50 days, when they attained an adult level in the testis. In contrast, GnRH pulse frequency was lower at 15 days (**Fig. 2**), indicating that an acceleration of pulsatility precedes the onset of puberty, in agreement with our previous findings (3). When the hypothalamic explants of 15-day-old rats were incubated without glycine, no GnRH release could be detected at any time during a 3-h experiment. Therefore, the interval between GnRH secretory pulses, if any, should have been greater than 180 min under those conditions (**Fig. 3A**). When glycine concentrations of 0.0001, 0.001, and 0.01 µmol/L were used, GnRH release became detectable, and secretory pulses were seen. The mean GnRH interpulse interval decreased, in relation to the glycine concentration (**Fig. 3**, **B** and **C**), to a mean of 61 min, which was observed using 0.01 µmol/L glycine (**Fig. 3D**). Using 0.1 µmol/L glycine, an hourly interval was seen as well (**Fig. 3E**). A further increase in the glycine concentration resulted in more frequent GnRH pulses that occurred at a significantly shorter interval using 10 µmol/L glycine (**Fig. 3G**) than using 0.1 µmol/L. Such an effect was not seen using 100 µmol/L glycine (**Fig. 3H**).

When explants from 15-day-old animals were incubated using 0.01 µmol/L glycine (**Fig. 4**, *upper panels*), the addition of the glycine antagonists 7CK (**Fig. 4B**) and strychnine (**Fig. 4C**) or the GABA agonist loreclezole (**Fig. 4D**) failed to affect the frequency of pulsatile GnRH secretion. When incubation was performed in a high (10 µmol/L) glycine concentration (**Fig. 4**, *lower panels*), the interval between GnRH pulses was reduced (**Fig. 4**, **E** *vs.* **A**). The acceleration of pulsatility caused by 10 µmol/L glycine was prevented by 100 µmol/L 7CK (**Fig. 4F**) or 10 µmol/L loreclezole (**Fig. 4H**). Using 100 µmol/L strychnine, the glycine-induced acceleration of GnRH pulse frequency was partially inhibited (**Fig. 4G**).

Using explants from 25-day-old rats under control conditions, the interpulse interval of GnRH secretion was shorter than that at 15 days (**Table 1**). Using explants from 25-day-old rats, loreclezole as well as vigabatrin increased the GnRH interpulse interval (**Table 1**); this was not the case for loreclezole using explants from 15-day-old animals (**Fig. 4D**).

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**Fig. 3.** Effect of glycine concentration in incubation medium of retrochiasmatic hypothalamic explants of 15-day-old male rats on the pulsatile secretion of GnRH. At each glycine concentration, a representative secretory profile of an individual explant and the mean (±SD) interval between GnRH pulses are shown. Four to six explants were studied in each condition. The *asterisks* denote a mean interval significantly different ($P < 0.05$) from that seen using 0.01 µmol/L glycine (A–D) or 0.1 µmol/L glycine (E–H). The *broken lines* indicate the limit of detection of GnRH release. ●, Significant pulse (Pulsar program).

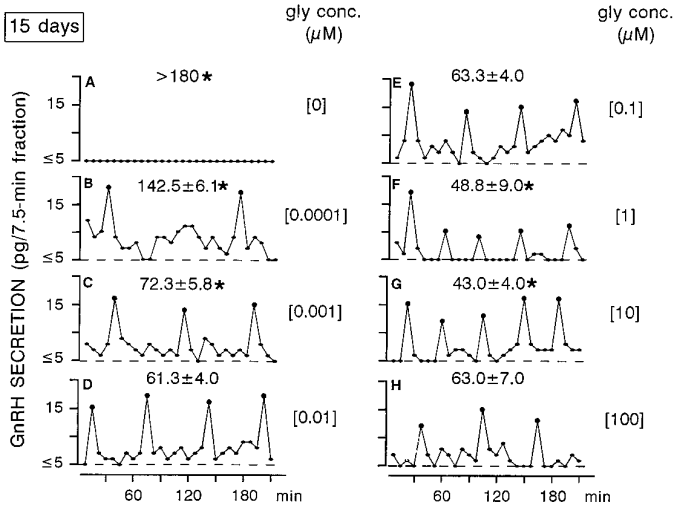


**Fig. 4.** Effect of the glycine antagonists 7CK and strychnine and the GABA agonist loreclezole on pulsatile GnRH secretion from retrochiasmatic hypothalamic explants of 15-day-old male rats incubated using low (0.01 µmol/L) or high (10 µmol/L) glycine concentrations. In each study condition, a representative secretory profile of an individual explant and the mean (±SD) interval between GnRH pulses are shown. Four to six explants are studied for each condition. The *asterisks* denote a significant difference ($P < 0.05$) between the mean intervals seen under control conditions and using the pharmacological glycine antagonists and GABA agonist. The *broken lines* indicate the limit of detection of GnRH release. ●, Significant pulse (Pulsar program).

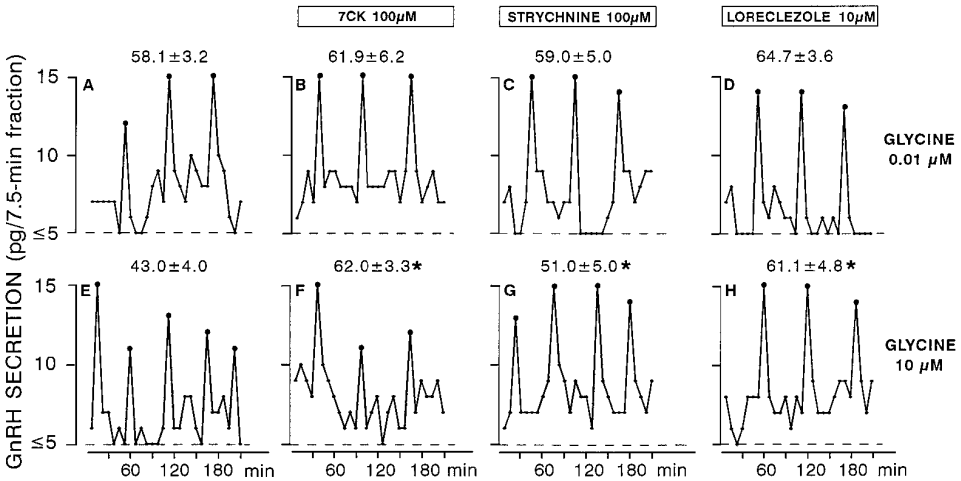

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**Table 1.** Effects of loreclezole and vigabatrin on the interval between GnRH pulses secreted by hypothalamic explants of 25- day-old male rats incubated using 0.01 µmol/L glycine.

| GABA agonists (10 µmol/L) | GnRH interpulse interval (min) |
|---|---|
| Control | 39.6 ± 4.9 |
| Loreclezole | 52.5 ± 3.5[a] |
| Vigabatrin | 48.8 ± 4.7[a] |

The data are the mean ± SD of the interval between GnRH pulses secreted by four to six individual rat hypothalamic explants in each group.
[a] $P < 0.05$ compared to 25 day controls.

# Discussion

The patient described has a neonatal form of NKH. This severe genetic disease results from an inherited defect in the enzymatic system cleaving glycine. In NKH, the cerebrospinal fluid concentrations of glycine are highly increased into a characteristic range of 83–280 µmol/L (30). Some NKH symptoms, such as hypotonia, involve the inhibitory strychnine-sensitive glycine receptors (30), whereas the pathogenesis of seizures involves the excitatory strychnine-insensitive glycine receptors belonging to the NMDA-receptor complex (31). In the reported girl, evidence of abnormal estrogenic effect starting early in postnatal life was provided by the acceleration of growth during the first year and the advancement of bone maturation. As commonly seen in female precocious puberty, breast tissue developed after growth acceleration. Although we have not documented the central origin of puberty through a GnRH test, basal LH was increased and later returned to normal when pubertal development regressed. CNS imaging did not show any anatomical abnormality in the hypothalamus or other regions known to be involved in the pathogenesis of precocious puberty.

Based on the experimental induction of precocious puberty after chronic NMDA administration (11, 12), the occurrence of precocious puberty in a patient with NKH was thought to result from excessive stimulation by glycine of the NMDA receptors linked to the GnRH neurons. The development of precocious puberty after NMDA receptor-mediated lesion of some neurons inhibitory to GnRH neurons cannot be ruled out. This hypothesis, however, is unlikely because neonatal overstimulation of glutamate receptors results in panhypopituitarism secondary to the loss of a majority of neurons in the arcuate nucleus (32), whereas there isno evidence of such deficiencies in our patient. Early sexual maturation has not been reported in NKH patients to date. It is possible that only some NKH patients have glycine concentrations that make them prone to early pubertal maturation. Indeed, only a particular range of glycine concentrations can potentiate the NMDA-evoked release of GnRH (25) and pulsatile GnRH secretion (this study), whereas such a potentiation is not observed using higher glycine concentrations. The concentrations of glycine commonly used for *in vitro* studies of hypothalamic explants are about 100–1000 times lower than CSF concentrations. The differences between *in vitro* and *in vivo* conditions make difficult any

comparison based on substrate concentrations, and interpretation of our data relies more on the relative changes in glycine concentration than on the absolute levels achieved.

The symptoms of precocious puberty regressed and finally disappeared, whereas there was no change in the metabolic and neurological situation. Spontaneous regression, which is the usual outcome of premature thelarche, was unlikely to occur in central precocious puberty. Therefore, we postulated that changes in antiepileptic treatment could have interfered. This hypothesis is consistent with the pivotal role of the $GABA_A$ (ionotropic) receptors in the prepubertal inhibition of pulsatile GnRH secretion in the female monkey (15). The $GABA_A$ receptor is a heteromer of different subunits with distinct binding sites for GABA, anesthetic or sex steroids, barbiturates, and benzodiazepines (33). Therefore, the $GABA_A$ receptor can be targeted directly by some antiepileptic drugs such as barbiturates, benzodiazepines, and loreclezole (22), or indirectly by drugs such as vigabatrin (23) or sodium valproate (16), which reduce GABA degradation. The widespread use of different pharmacological GABA receptor agonists in NKH may be an additional reason why precocious puberty has not been commonly seen in those patients. The inhibition of pubertal development by GABA agonists in our patient is consistent with the inhibition of LH secretion by valproate seen in peripubertal subjects (17). In adult women, however, such an inhibition could not be observed (34, 35), in agreement with our finding that the GABA agonist muscimol and the antagonist bicuculline did not affect pulsatile GnRH secretion in the postpubertal rat hypothalamus at 50 days of age (14). These late developmental changes in GABA receptor-mediated effects may be related to sex steroids, because acute inhibition of pulsatile LH secretion by valproate was found in postmenopausal women (19).

As it was not ethically possible to evaluate the role of NMDA receptor-related glycine receptors and $GABA_A$ receptors in the affected child, we tested the proposed concept in a well characterized paradigm of rat hypothalamic explants. The immortalized GnRH neurons are an alternative contemporary model. However, developmental changes cannot be studied in these conditions using hypothalamic explants (2, 3). In addition, the retrochiasmatic hypothalamus appears to contain a pulse generator different from the GnRH neuron (24) and involving both the facilitatory NMDA component and the inhibitory $GABA_A$ component (13, 14), which are known to exist in the subhuman primate as well (12, 15). The glycine-dependent increase in the frequency of pulsatile GnRH secretion is mediated at the NMDA receptor, as this effect is prevented by 7CK, an antagonist specific for the glycine-binding site of the NMDA receptor complex (26). However, 100 µmol/L of the antagonist 7CK does not abolish pulsatile GnRH secretion. Using a higher 7CK concentration of 500 µmol/L, which is able to completely suppress GnRH secretion evoked by NMDA (14), the frequency of pulsatile GnRH secretion is reduced, but the secretion is not suppressed, as seen in the absence of glycine. These data suggest that glycine may contribute to GnRH secretion through different mechanisms, one of which involves NMDA receptors. When glycine or normal development results in accelerated GnRH pulsatility, loreclezole or vigabatrin can restore a prepubertal pattern of pulsatile GnRH secretion. These findings are in agreement with the effects of bicuculline, a typical pharmacological antagonist of $GABA_A$ receptors (14, 15).

In summary, the premature development of puberty in a female infant with NKH and the regression of those manifestations during therapy with GABA agonists provide preliminary clinical evidence of involvement of NMDA and GABA receptors in the facilitatory and inhibitory control of the onset of human puberty. These data point to the pathophysiological importance of extensive evaluation of neuroendocrine function in NKH as well as in peripubertal patients treated with GABA agonists as antiepileptic agents.

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