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Neurotoxicology and Amino Acid Intake During Development: The Case of Threonine

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CASTAGNE, V., J.-C. E. MAIRE AND M. GYGER. *Neurotoxicology and amino acid intake during development: The case of threonine.* PHARMACOL BIOCHEM BEHAV **55**(4) 653–662, 1996.— The development of the central nervous system is highly dependent on an adequate supply of nutrients. In particular, protein and amino acid availability is of major concern during gestation and in early postnatal life. Numerous data have been published on some amino acids directly involved in brain functions as neurotransmitters or indirectly as precursors of neurotransmitters, but scant information is available on the possible consequences of hyperthreoninemia, a phenomenon repeatedly noted in clinical reports. The results of neurochemical and behavioral studies in the developing rat suggest that despite numerous possible effects of threonine on brain constituents, moderate hyperthreoninemia does not impair markedly the development of the central nervous system. **Copyright © 1996 Elsevier Science Inc.**

Amino acid intake Threonine Rat Behavior Ontogenesis Brain Glutamate Glycine Microdialysis

Ontogeny of the Central Nervous System as a Critical Period for Neurotoxic Effects

Ontogeny is a period of high susceptibility of the central nervous system (CNS) toward its environment. Numerous chemicals (e.g. methylmercury, ethanol) or physical phenomena (radiation) may induce classical teratologic defects such as gross malformations of the brain or functional neuroteratologic abnormalities including behavioral or psychologic defects (120,129). The complexity of cellular mechanisms involved in CNS development is reflected by the numerous potential sites of action of teratogenic perturbing events (66,120). The teratogenic effects of *in utero* and neonatal ethanol exposure are exerted through various possible targets in the CNS, one of the best known being the glutamatergic transmission (96). Exogenous teratogens are not the only factors able to interact with the developing CNS. The very high metabolic activity underlying the intense transformations of nervous tissue (66) explains the importance of adequate nutrient levels for normal CNS ontogeny.

In human subjects, inadequacies in nutritional intake of the pregnant mother or of the young infant is a potential risk factor for brain development (78). The concept of early malnutrition is very imprecise and it seems more adequate to study the consequences of more specific nutritional imbalances (131). Nevertheless, the relationships between early malnutrition and CNS development are difficult to study since the psychosocial environment of the infant may compensate for the effects of malnutrition (131).

Amino Acid Intake as an Essential Requirement for CNS Development

Amino acid intake is a major environmental factor in CNS ontogeny and imbalances of some amino acids such as phenylalanine (Phe) or tryptophan (Trp) have dramatic and long-lasting consequences on brain functions (47,48,130). When considering the possible consequences of an imbalance in a particular amino acid, one may distinguish direct and indirect actions of this nutrient (93). Direct effects may occur for amino acids used as neurotransmitters in the CNS, such as glutamate (Glu) or glycine (Gly). Indirect effects may be present for amino acid precursors of neurotransmitters (see below). Oth-

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erwise, indirect effects are principally related to entry into the brain. Various carriers facilitate the passage of amino acids through the blood brain barrier (BBB) (82,85). Competition between amino acids to gain access to their carrier explains why an excess in a particular amino acid results in a lower entry of its competitors, and, in a deficiency in the functions normally related to those amino acids. Beside competition at the BBB level, amino acids also compete for entry into brain cells through specific uptake systems (18,87).

Phe intake has been extensively studied in the context of hyperphenylalaninemia and phenylketonuria (46,49). One of the deleterious mechanisms of action of excess Phe may be through protein synthesis perturbation (50). Phe could also diminish Trp entry into the brain since the two amino acids compete for the same carrier across the BBB (45). Finally, Phe may be converted into tyrosine (Tyr) in the liver, which may influence catecholamine synthesis (68).

Trp availability has an influence on serotonin synthesis (29,45,48). Serotonin is a major neurotransmitter in the CNS and influences various phases of CNS ontogeny, including the very early phases (25,64). Since early perturbations of CNS ontogeny have more drastic teratogenic consequences as compared to late perturbations (120), it is not surprising that Trp availability is a major factor in brain growth (47). Trp availability has been shown to affect various psychobiological parameters in the human adult (137). These considerations motivated the design of clinical studies in formula-fed infants (27,138). The consumption of formulas enriched in Trp is associated with shorter sleep latencies (117).

Tyr intake influences catecholamine, especially dopamine, brain concentrations (101). In conditions of high metabolic activity (e.g. stress), Tyr availability may be a limiting factor in the activity of dopaminergic midbrain neurons.

Histidine (His) also enters into the brain predominantly using the carrier for large neutral amino acids (83). His availability regulates, to a large extent, the synthesis of histamine in the brain (26). Histaminergic transmission is less well understood than catecholaminergic transmission but has important effects on neurologic activity (113), suggesting that His intake is a critical parameter for CNS functions.

Clinical studies have repeatedly shown that human infants fed formulas containing bovine milk proteins (especially with a high whey/casein ratio) have elevated threonine (Thr) intake and threoninemia as compared to breast-fed infants (3,43, 53,54,67,91,94,97). The consequences of high blood Thr concentrations have been questioned by some pediatricians (97). Nevertheless, very few data are available today regarding the consequences of increased plasma Thr. In adult human subjects, oral administration of large doses of Thr has been used without adverse secondary effects as a tentative therapy for spastic syndromes (38). In adult rat, a large excess of dietary Thr was shown to be weakly toxic as compared to other amino acids (109). In the weanling rat, a large excess of Thr reduced weight gain and food intake, depending on the protein content of the diet (79,107). In the growing pig fed 20% protein diets, excesses up to 4% Thr reduced weakly weight gain and food intake (20,22). The growth of 8-days-old chicken fed 23% protein diet was moderately impaired by excesses of Thr (21). Overall, these scarce data suggest that dietary Thr may hardly interfere with brain development. Nevertheless, a rare metabolic disorder has been described in a male infant having spontaneous hyperthreoninemia (95). Elevated blood Thr was associated with convulsions and growth perturbations. Unfortunately, this disease has not been identified in other subjects and its specific effect on brain development is still unknown.

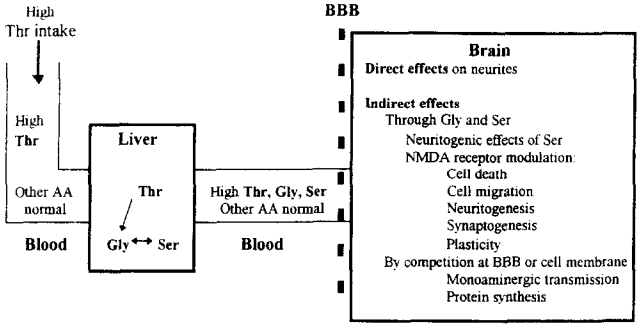

FIG. 1. Potential sites of action of Thr on CNS development. See text for comments. AA: amino acids, the term monoaminergic transmission includes catecholamine, serotonin, and histamine containing neurons.

The chronic effects of excess Thr intake on CNS development are thus still unknown.

Possible CNS Targets Influenced by Thr Availability

High dietary Thr intake increases plasma and tissue Thr concentrations (55,136). In the liver, Thr can be converted into Gly by Thr dehydrogenase (6). In the brain, as well as in other tissues, Gly can be transformed into Serine (Ser) by serine hydroxymethyltransferase (74). Thr is efficiently transported through the BBB (125). It has been shown that intraperitoneal injection of Thr in the adult rat enhances Gly concentrations in the brain (69). It is probable that amino acid transport into the brain is even more important in the foetus or the preweanling rat whose BBB does not totally mature before postnatal day 24 (112).

It is probable that Thr has few, if any, direct effects on the CNS. It has been shown that injection of Thr into the prepyriform cortex influences the voluntary food intake of the rat. Nevertheless, this effect was not dose-related and probably reflected the fact that Thr was the limiting dietary amino acid (5).

The possible effects of Thr on the brain are most likely exerted indirectly through the actions of Gly or Ser (Fig. 1). Gly is a neurotransmitter involved in various aspects of brain functions (2). Nonketotic hyperglycinemia is an often lethal metabolic disease characterized by failure of the glycine cleavage system and increased tissue Gly concentrations (122). Infants affected by this disease have significant neurologic symptoms often leading to coma and death. The rat has been successfully used as an animal model to show that the abnormal neurologic development is directly related to excessive brain Gly concentrations (17). Ser is not a neurotransmitter but is able to modulate CNS functions and development. Recently, it has been shown in vitro that L-Ser enhances the neuritogenesis of chicken embryonic dorsal root ganglion neurones (110). Nevertheless, this effect occurs at concentrations lower than normal tissue concentrations and is probably not involved as a regulatory mechanism of neuritogenesis in the context of in vivo development. A more likely mechanism of action of Gly and Ser on CNS ontogeny is through modulation of NMDA receptors for Glu (103). Glu has widespread actions in the CNS (80) and in particular in brain development (73,75,76,111). It has been shown that Glu influences neuronal survival (11,36,37) and migration (60), neurite growth (11,12,56), and establishment of synaptic connections (92).

Moreover, the development of the visual system and plasticity in the visual cortex are related to Glu neurotransmission (32,59,62).

Increased blood threonine could also affect CNS development through competition with other amino acids for transport across the BBB (123). A decrease in Tyr entry into the brain could lead to a drop in catecholamine synthesis, whereas impaired Trp entry could reduce serotonin synthesis (30,45). Moreover, a perturbation of His transport through the BBB may interfere with histaminergic neurotransmission (77). More generally, it is conceivable that a modification in amino acid availability to the brain may decrease CNS protein synthesis (63).

An Animal Model of Hyperthreoninemia

The above description of some of the possible consequences of excess Thr intake indicates the existence of a neurobiologic basis for developmental effects of Thr. Neurochemical effects of a substance on the developing CNS are important reflections of a potential neuroteratogenic action (120,129). Thus, we decided to study the consequences of excess dietary Thr beginning during pregnancy in a rat model of hyperthreoninemia. In animal studies, the influence of psychosocial factors on brain development are less pre-eminent than in humans, suggesting that the study of developmental consequences of nutritional inadequacies are more specific in animal models (78). Beside gross malformations present at birth, modern neurotoxicological studies include more discrete developmental landmarks (52,120,124,128,129). It is now widely accepted that in order to study the potential neurotoxic effects of a compound, it is important to include both neurochemical and behavioral investigations (129).

Our studies in the field of hyperthreoninemia were done to complement the minimal data available from humans or animals. We did not try to perform a complete neuroteratological analysis but put our efforts on specific markers of CNS development, especially on markers susceptible to modifications by Thr intake according to the scheme presented in Fig. 1. First, we characterized some of the neurochemical correlates of high Thr intake in the young postweaning rat, including intra- and extracellular amino acid concentrations in the brain and other tissues (14,16). Second, we performed a study of behavioral development of rats hyperthreoninemic since conception (15). A number of important developmental landmarks were not studied, for example the histologic characteristics of the brain, performance in learning and memory tests, and sexual and social behaviors.

ANIMAL STUDIES

Experimental Design

Neurochemical correlates of high dietary Thr were performed in young postweaning male rats (see Fig. 2A). In the first study (16), we described the effects of three dietary Thr supplements (1.5, 2.0, and 15 times normal Thr intake) on plasma, liver, muscle, and intracellular brain amino acid concentrations. Rats received the various diets for two weeks and were then killed by decapitation. In a second study (14) we used two dietary supplements of Thr (2 and 8 times normal Thr intake). Brain and spinal cord were dissected out and the extracellular concentrations of amino acids were studied in vitro by the microdialysis technique (126).

Morphologic and behavioral development were studied until 90 days of age in rats exposed to high Thr since conception

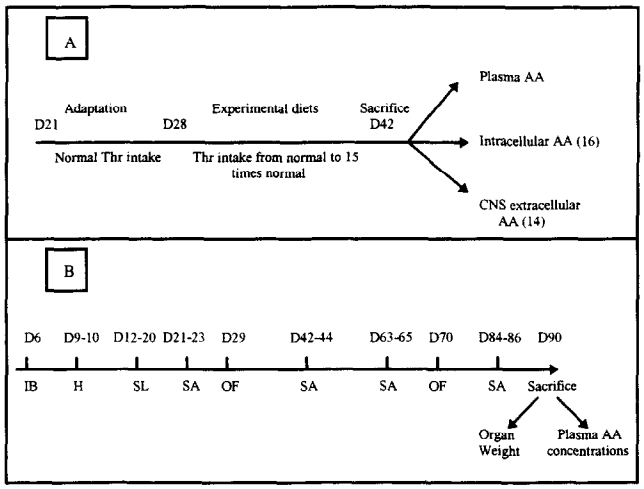

FIG. 2. A. Experimental design for neurochemical studies. D means postnatal day of investigation. Rats received standard diets during the first week of adaptation until D28. Thereafter, they received the experimental diets (see text for composition) until D42. For the study of intracellular AA concentrations, Thr intakes were 1.0, 1.5, 2.0, and 15 times the normal intake. For extracellular AA concentrations, intakes were 1.0, 2.0, and 8.0 times normal. B. Experimental design for the study of behavioral ontogeny. Days of behavioral testing are indicated above the horizontal line and the nature of tests under the line. IB: independent ingestive behavior, H: homing behavior, SL: spontaneous locomotor activity, SA: spontaneous alternation, OF: open field. In addition, rats were weighed every 4 days from birth to weaning and weekly thereafter.

(15). We studied the effects of moderate to high increases of Thr intake, 1.7 and 4 times normal Thr intake (see (15) for absolute values which change as a function of age). Moreover, we attempted to detect the critical period for the effects of Thr on brain by comparing the development of rats exposed to moderate Thr intake from conception to birth and then from weaning until sacrifice at day 90. These rats differed from the preceding group as they received a normal Thr intake during lactation. We defined four groups of animals: the first group received normal Thr intake from conception until death at 90 days, the second group received moderate (1.7 times normal intake) Thr from conception until weaning, the third group received moderate intake from conception until death, and the last group received high Thr intake (4 times normal intake) from conception until sacrifice.

The Thr supplementations were calculated as a function of the amounts observed in breast-fed or formula-fed human infants. We intentionally chose the highest ratio (1.7) of Thr concentrations in formula over Thr concentrations in human milk to define our moderate intake in rats. Afterward, pup development was studied from birth until 90 days, according to the experimental design shown in Fig. 2B. In order to minimize the number of animals, we assumed that a continuous follow-up of body weights and a detailed analysis of organ weights on day 90 would be sufficient to detect gross malformations caused by high Thr intake.

In all studies, amino acid concentrations were measured in plasma obtained after decapitation, in order to verify the extent of hyperthreoninemia associated with our diets and of hyperglycinemia secondary to the hepatic conversion of Thr into Gly.

Chemical and Neurochemical Correlates of High Thr Intake

Plasma amino acid concentrations. In the postweaning rat, high Thr intake for two weeks induced a dose-related hyperthreoninemia (14,16). Comparable data have recently been published in the growing rat fed 8 or 15% protein diets enriched with 0.5 to 4% free Thr (107). Rats fed 1.5 times the normal Thr intake displayed slightly higher plasma Thr concentrations (+38% as compared to Thr concentrations of rats receiving a normal diet). In rats fed 2 times the normal intake, plasma Thr concentrations were more consistently increased (+138% and +105% in (14,16), respectively). With high Thr intake (8 times normal), plasma Thr rose drastically (+1962% in (14)). In the longitudinal study of morphologic and behavioral development, moderate Thr supplement (1.7 times normal) induced, at day 90, an hyperthreoninemia between +42% and +69% as compared to control pups, depending on the sex of the animals and the protocol of Thr supplementation (15). Hyperglycinaemia was observed in rats fed 4 to 15 times normal Thr intake, illustrating the conversion of Thr into Gly (6). Plasma Ser concentrations were inconsistently modified by Thr intake. Increases in plasma Gly and Ser concentrations have been described in postweanling rats fed 8 or 15% protein diets containing up to 4% excess Thr (107). In the growing pig, excess dietary Thr increased plasma concentrations of Thr, Gly, and Ser (20).

Brain intracellular amino acid concentrations. Moderate to high dietary supplement of Thr (2.0 and 15 times normal) raised Thr concentrations in various CNS structures including spinal cord, cerebral trunk (which comprises mes-, myel-, and metencephalon, but not cerebellum), hypothalamus, and cortex (16). Postweanling rats fed 8 or 15% protein diets supplemented with free Thr during 8 days had a dose-related increase in Thr concentrations in the brain (107). Lower Thr intake (1.5 times normal) only raised Thr concentrations in the cortex. Gly concentrations were enhanced by very high Thr intake (15 times normal) in some structures including cerebral trunk and cortex. Lower Thr intakes inconsistently modified Gly CNS concentrations. Ser concentrations were weakly modified by the diets. In accordance with our results, a rise in Gly concentrations has been described in the spinal cord after intraperitoneal injection of Thr in the adult rat (69). The evolution of Gly intracellular concentrations after Thr dietary intake or systemic treatment shows that manipulations of Thr plasma concentrations in the pharmacologic range could disturb brain neurochemistry, in accordance with the scheme presented in Fig. 1. Nevertheless, moderate intakes, such as those brought by common diets, including cow milk whey-based formulae, did not seem to interfere with glycinergic neurotransmission (16). Moreover, in another study, postweanling rats fed Thr-enriched diets for 8 days had no changes in their brain amino acid (other than Thr) concentrations (107).

Brain extracellular amino acid concentrations. In vitro extracellular Thr concentrations were raised in cortex, cerebral trunk, and spinal cord of rats fed 3.3 times the normal Thr intake (14). Nevertheless, Gly extracellular concentrations were not modified, suggesting that some regulatory mechanisms maintained normal Gly concentrations near its receptors. Export through BBB, reuptake of extracellular Gly (2), or metabolism through the Gly cleavage system (108) could be potential mechanisms explaining the preservation of normal Gly extracellular concentrations despite the intracellularly observed increase (16). Thr intake did not modify the extracellular concentrations of aspartate, a putative neurotransmitter (44,84), or of Glu (14).

In conclusion, neurochemical data suggest that moderate increases in Thr intake could probably not modify brain amino acid neurotransmission. Thr concentrations may be increased after high Thr intake in plasma and brain intra- and extracellular compartments and Gly concentrations may be raised in plasma and intracellular brain compartments. Nevertheless, extracellular concentrations of Gly, aspartate, and Glu are not modified by dietary Thr.

Effects of High Thr Intake on the Morphologic Development of the Rat

In the young postweaning or adult rat, excess dietary Thr has been shown to be weakly toxic, slightly decreasing growth and food intake (79,86,109). Under our conditions, 15 times normal Thr intake in the postweaning rat reduced body weight (−13% as compared to rats receiving a normal intake) after two weeks of treatment (16). In a more extensive study of morphologic development (15), high Thr intake (4 times normal) beginning from conception until death at 90 days markedly decreased body weight at all ages. Our results are consistent with the notion that a toxic effect has a higher probability of being expressed if it is present early in development (120,129). The body weight on day 2 (first day of measure) was also reduced in pups receiving high Thr intake. Thereafter, those rats began to grow but never reached the weights of pups fed moderate or normal amounts of Thr. It has recently been shown (107) that excesses of dietary Thr above 1% of the diet content induced noticeable body weight reduction. In this context, the rat seems as sensitive as the chick to the effects of Thr (21), the weight gain of these two species being much more impaired by high Thr intake than that of the pig (20,22). The excessive consumption of an amino acid induces an imbalance of amino acid intake, resulting in various biochemical adaptations, including reduced protein synthesis, and finally in reduced weight gain (109). Beside the general effects of imbalance, some amino acids, such as methionine, show additive specific toxic effects which further decrease weight gain (79,109). The decreased weight gain of rats fed high amounts of Thr (14,15,79,107) is moderate as compared to the effects of pup undernourishment during gestation and lactation (33,72,102) and is low as compared to the effects of diets containing high concentrations of methionine (79,109). These data suggest that dietary Thr mainly affects body weight through an effect of imbalance of amino acid intake and not through additional specific toxic effects.

Organ weights were not consistently modified by an increase in Thr intake. These data have been recently reproduced (107). Kidney weight (relative to body weight) was diminished (−12%) specifically in females receiving moderate (1.7 times normal) Thr intake but was unchanged when Thr intake was 4 times normal. Relative brain weight was increased in females receiving 1.7 times normal Thr intake (+10%) and in females and males receiving a high (4 times normal) Thr intake (+7% and +8%, respectively). Severe protein malnutrition of the pregnant and lactating mother markedly impairs brain development as assessed by weight, phosphoprotein, and phospholipid content (71). In comparison with the drastic effects of severe protein malnutrition, high dietary Thr has little effect on brain weight, but, specially during lactation, mainly effects body weight. On the whole, cerebral development did not reflect gross abnormalities associated with moderate or high dietary Thr intakes (15,107).

Effects of Thr Intake on Behavioral Development, a Longitudinal Study

Independent alimentary behavior. The existence of a form of alimentary behavior distinct from suckling and independent from the mother can be demonstrated in the preweaning rat pup (42). The young rat separated from its mother and fasted for a few hours engages in an active ingestive activity if placed in the presence of food or if orally infused with liquid diets. The motor sequences involved in independent alimentary behavior are different from the neuromuscular activity associated with suckling. The intero- and exteroceptive cues modulating the two behaviors are also different. Moreover, previous experience with suckling is not necessary for the expression of independent alimentary behavior. Various experimental results suggest that independent feeding is the real precursor of the adult feeding pattern (39). The end of consummatory activity (satiety) has a complex regulation (121). At 6 days, the main factor inducing the end of alimentary intake is gastric distention (41,90). At 15 days, satiety is also principally governed by gastric distention but postgastric factors begin also to modulate feeding (88,89). The neural substratum involved in the regulation of independent alimentary behavior has been studied by the technique of 2DG autoradiography (40). The motivational, sensory, and motor aspects of the alimentary behavior involve different, although sometimes overlapping, neural structures.

In the group of rats whose mothers consumed diets containing 1.7 times more Thr than normal amounts, but whose pups received a normal Thr intake from birth until day 6 (day of testing), an enhancement of sucrose consumption was clearly observed in comparison with the other groups (15). Nevertheless, this effect was not found in pups receiving moderate (1.7 times normal) or high (4 times normal) Thr supplements from conception until testing. The higher sucrose intake of pups supplemented with Thr during gestation but not lactation cannot be interpreted as a sign of nutritive deficiency since independent alimentary behavior at day 6 is solely regulated by gastric distention (41,90). The fact that the pups in the four groups studied consumed significant amounts of sucrose (between 1 and 2% of their body weight during the 30 min. of testing) suggests that the CNS substratum involved in the complex phenomenon of independent ingestive behavior (40) is functional in pups exposed to a high Thr intake during gestation and the first week of lactation (15).

Homing behavior. Homing, as defined by the activity developed by a pup to find its mother, is a form of approach behavior and requires orientation skills. Homing appears on day 2 and matures between days 4 and 10 (114,133). Homing behavior is highly dependent on the capacities of olfactive discrimination of the pups. If the nest odor is modified by exogenous odors, the preference of the young rat is modified in favour of the novel odor (70). The odor of the mother can be modified by dietary manipulations which also induce modifications of the olfactive preferences of the young (119). Homing behavior is also influenced by circadian rhythms. During the dark phase of the light cycle, motor activity is elevated, inducing an augmentation in homing performance (51).

We measured homing behavior using the multipup homing protocol (133). Performance in the homing test was independent of dietary Thr intake (15). The only effect observed was that pups receiving high Thr intake (4 times normal) from conception until testing on days 9 and 10 displayed a short latency to leave the start area in the test apparatus when the mother was absent from the goal area. This effect appeared to be of little significance since it was observed only on the first day of testing and was never observed in the presence of the mother (15). It has recently been shown that rat pups whose mothers were subjected to protein undernutrition during gestation displayed a specific impairment of homing behavior (33). Our results show that moderate or high Thr excess does not impair homing behavior, despite the fact that early malnutrition does. We cannot infer from our data that high Thr intake is absolutely harmless toward the neural processes involved in recognition of the mother and in orientation toward the goal area. Nevertheless, the normal performance in the homing test suggests that the development of these processes is largely preserved from the possible harmful effects of Thr.

Spontaneous locomotion in a non- or weakly stressful environment. A detailed analysis of locomotor behavior shows that during the first postnatal week, the rat performs inadequate movements, principally torsions of the body without coordinated movements of the legs (1). Crawling appears during the second week and real walking at about day 15 (8,134). The first signs of exploratory activity are observed at the same time and show a peak of intensity on day 20. Exploration diminishes thereafter (8). The acquisition of posture skills is intimately related to the ontogeny of locomotor activity (34), and to brain development (81,118). The rise in activity observed in very young rats is associated with the maturation of pontine (e.g. reticular formation) structures involved in arousal. On the contrary, the diminution of the level of activity is related to the development of higher brain structures (e.g. cortex) which inhibit the lower brain systems (13). The motor capacities of rat pups develop concomitantly with hippocampal electrical activity (65).

Our results showed that Thr intake did not modify the development of adult-like walking and rearing, nor the intensity of locomotor activity (15). Early protein undernutrition of the pregnant mother disrupts various components of motor activity in pups observed between days 29 and 100 (72). Important modifications of postweaning and adult motor activities are induced by manipulations of Tyr intake of the pregnant mother (99,105). The development of CNS dopaminergic neurotransmission has profound effects on the ontogeny of locomotion and on adult motor activities (57). The normal motor development of Thr loaded rats during gestation and lactation (15) suggests that high Thr intake does not significantly modify the ontogeny of dopamine containing motor systems in the CNS. Although we did not perform an extensive study of motor skills (61), our data suggest that Thr intake does not markedly disturb the development of the complex nervous systems involved in spontaneous locomotion. Nevertheless, it remains possible that more sensitive methods of measure of motor performance (57) could detect subtle effects of dietary Thr. Our behavioral data are consistent with previous neurochemical data (14), suggesting that despite the influence of Thr intake on Gly concentrations in CNS tissue (16,107), CNS extracellular Gly concentrations are not modified, in particular in the spinal cord where Gly is involved in motor activity (2). Moreover, extracellular Glu and Asp concentrations in the spinal cord were not modified by Thr intake (14), further strengthening the evidence that supraspinal excitatory and intrinsic spinal inhibitory afferents to motoneurons are not drastically modified by dietary Thr.

Locomotion in a stressful environment. In the open field test, 15-day-old rats maintain a stable exploratory activity level during the entire test sessions. On the contrary, 21-day-old rats are very active at the beginning of the test session but

very quickly stop their exploratory activity in a manner comparable to the adult pattern of habituation (10). This phenomenon is consistent with the development of head poking activity which is not subject to habituation before 25 days (28). In the open field, rearing appears on day 15 and increases progressively thereafter. Adult level of activity is not reached before day 30 (104). It is possible to accelerate the ontogenesis of rearing by precocious eyelid opening (31). Exploratory activity in the open-field is dependent on arousal (135). Two components motivate the exploratory behavior of the rat in the open-field: the exploratory drive and the emotional reactivity. The measures performed in our conditions are a reflection of both components. Although invariant relationships between behavioral markers of exploratory activity and neuroendocrine or neurochemical stress responses have not been defined (7), it is clear that activity in the open-field test is a good index of fear and fearfulness (135). The links between open-field activity and neuroendocrine stress responses are further confirmed by similarities in their ontogenic patterns. During the first three weeks of life, the stress response of the hypothalamo-hypophyseal axis is weaker than in the adult (106,132). This hyporesponsivity is not related to an hypoactivity of the endocrine system but reflects probably a weak transduction of the stress signal into neurosecretion of CRF from the hypothalamus (132).

Exploratory activity during the open-field session on day 29 was not modified by Thr intake (15). On day 70, the number of rearings was slightly increased (34 %) in rats receiving 4 times the normal Thr intake from conception until testing as compared to normal-fed rats. Other parameters of activity were independent of Thr intake. Young and adult rats exposed prenatally to haloperidol display an increase of locomotor activity in the open field test (115), showing that this test can detect abnormalities in the development of the dopaminergic system. Our results showed that Thr intake did not markedly influence exploratory activity in a stressful environment: there is thus no evidence suggesting that Thr intake could impair dopamine neurotransmission in the brain or the development of CNS systems involved in the control of emotions and stress responses (7).

Spontaneous alternation in a Y maze. This behavior is highly dependent on visual cues and on the capacity to orientate in the maze with a spatial strategy. It is possible to accelerate the development of spontaneous alternation by surgical opening of the eyelid on days 6-8 (31). The ontogenesis of the hippocampal formation is the major neurological event governing the appearance of spontaneous alternation (19,58). The experimental design used to study spontaneous alternation has important effects on the subject's performances. In designs based on simple and short sessions, adult levels of alternation are reached at about day 80 (58). On the other hand, when rats are observed in long sessions, alternation is observed earlier (9,35). The maturation of spontaneous alternation is intimately related to the ontogenesis of the cholinergic central system (24). Before 24 days, the alternation rate is not modified by experimental manipulations of the cholinergic system. After 24 days, antagonists of muscarinic acetylcholine receptors diminish the alternation rate whereas drugs enhancing cholinergic activity have the opposite effect (24). At 15-16 days, rats alternate below the level of chance and the alternation rate raises quickly to 75% on day 30, and to near 90% at day 100 (23). The tendency of the young to persevere in their initial choice does not result from poor memory capacity or from excessive arousal (23). The young rat is influenced by the same spatial and olfactory cues as the adult, but the young uses the cues to persevere in its arm choice whereas the adult uses the cues to alternate. It has been recently shown that performances in alternation result from the competition between the early tendency of rats to display a preference for a side of the maze (lateralization) and the late appearing alternance behavior, motivated by the tendency to explore the newest arm (98).

Whatever their Thr intake, rats displayed a normal ontogeny of spontaneous alternation (15), indicating no evidence for a marked effect of Thr on the development of the hippocampus or of the cholinergic central system. Whether more subtle effects of Thr intake on learning and memory performance could be detected using other tests remains to be established.

Grooming behavior. We measured this activity concomitantly to other behaviors during the tests of spontaneous locomotion (between days 12 and 20) and of open-field (days 29 and 70). The first signs of grooming are observed during the second postnatal day (8). The adult form of grooming is not displayed before day 21. The behavior develops progressively and an acceleration of the intensity of grooming is observed on the second week of life (4). Genital grooming is first observed on day 14 and peaks on day 44 (4).

Between days 12 and 20, pups receiving moderate to high Thr intake had some modification of their time spent on grooming (15). Rats receiving 1.7 times the normal Thr intake during gestation but not during lactation had a slight reduction of their grooming duration measured on day 12 as compared to control pups receiving a normal Thr intake. On day 18, rats receiving 1.7 and 4 times the normal Thr intake from conception until testing had an enhancement of grooming activity. These differences were not reproduced on the other days of testing, suggesting that Thr did not induce a profound modification of grooming behavior, as measured under our conditions. During the open-field test at day 29, rats receiving 1.7 times the normal Thr intake from conception until testing spent more time grooming than the other groups of rats (15). Once again, no clear effect of Thr could be deduced from these observations since the other Thr supplemented rats did not display the same tendency. Moreover, grooming duration was not affected by Thr intake during the open-field test at day 70. The discrete modifications observed in grooming activity during the first days of our longitudinal study are reminiscent of the irregular development of this behavior (8). Strong undernutrition of rat pups during lactation strongly enhances grooming activity from weaning until, at least, 60 days of age (102). The inconsistent and non-reproducible effects of Thr on grooming activity suggest that the development of the nervous structures involved in the control of this behavior (100,116) is normal in Thr supplemented rats. Grooming behavior has been related to numerous biological functions, including the regulation of the state of arousal (116) or the consequences of stress responses (127). The strong relationships between the intensity of grooming behavior and arousing conditions such as stressors and the fact that intracranial injection of adrenocorticotropic hormone induces grooming (116) suggest that the measure of grooming activity is related to the state of arousal. The weak effects of Thr on grooming activity are thus in accordance with our observations of exploratory activity in the open-field, thus giving no evidence that Thr intakes 1.7 or 4 times above normal could interfere with the nervous structures involved in the control of emotions.

CONCLUSIONS

A summary of Thr effects on the behavioral development of the rat is presented (Table 1). We conclude that moderate

TABLE 1

SUMMARY OF Thr EFFECTS ON BEHAVIORAL DEVELOPMENT OF THE RAT

	Postnatal Day				
Dietary Thr Content	6	9–10	12–20	29	70
1.7 times normal G and PW	Raised food intake		Reduced grooming		
1.7 times normal G-L-PW			Raised grooming	Raised grooming	
4 times normal G-L-PW		Reduced start-latency for homing	Raised grooming		Raised rearing

G: Gestation, L: lactation, PW: postweaning period. Normal Thr dietary content was defined for preweaning pups on the basis of rat milk amino acid composition (17) and for postweaning rats on the composition of standard diets (NAFAG 850 until day 50 and NAFAG 890 thereafter). See text for details.

or high intake of Thr (1.7 or 4 times normal) during and after gestation does not significantly impair the behavioral ontogenesis of the rat. Sensory, motivational, and motor components of various behaviors developed independently of the Thr intake of the pups. One could object that we did not measure a large enough behavioral repertory to prove the innocuous nature of excess dietary Thr, nevertheless, we did not find any consistent tendency for a Thr effect in our battery of tests and thus did not investigate further. For example, we did not study the ontogeny of memory and learning which are now widely included in neuroteratological studies (120,129). The spontaneous alternation scores of Thr supplemented rats did not suggest any gross defect in the ontogeny of the hippocampus and the cholinergic system which are essential nervous structures for the expression of spontaneous alternation. Nevertheless, it remains possible that extensive behavioral studies could reveal more subtle effects of dietary excess of Thr. The consistency of biochemical data, including intracellular amino acid concentrations in the brain and other tissues (16,107) and extracellular amino acid neurotransmitter concentrations (14), with behavioral observations (15) strengthens the assumption that Thr intake does not impair the development of the neurobehavioral parameters studied. This does not allow us to totally exclude the possibility that large excess of dietary Thr could have some neuroteratological effects. One should always be cautious when extrapolating from animal studies to the human situation, nevertheless, we think that the answer to the questions of some pediatricians (97) is that the moderate excess of Thr measured in some infant formulae as compared to human milk does not represent a clinical problem.

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