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Differential effects of developmental hypo- and hyperthyroidism on acetylcholinesterase and butyrylcholinesterase activity in the spinal cord of developing postnatal rat pups

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ABSTRACT

The plasticity and vulnerability of the rat spinal cord (SC) during postnatal development has been less investigated compared to other CNS structures. In this study, we determined the effects of thyroid hormonal (TH) deficiency and excess on postnatal growth and neurochemical development of the rat SC. The growth as well as the specific and total activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) enzymes of the SC were determined in hypo- and hyperthyroid rat pups at postnatal (P) days P1, P5, P10 and P21 (weaning), and were compared to age-matched untreated normal controls. AChE is a cholinergic synaptic enzyme while BuChE is a metabolic enzyme mainly found in glial cells and neurovascular cells. The SC is rich in somatic motor, autonomic cholinergic neurons and associated interneurons. Daily subcutaneous injection of pups with thyroxine (T4) and administration of antithyroid goitrogen propylthiouracil (PTU) in the litter's drinking water were used to induce hyper- and hypothyroidism, respectively. Enzyme assays were carried out spectrophotometrically at the above-mentioned ages, using SC homogenates with acetylthiocholine-chloride as the substrate, together with specific cholinesterase inhibitors, which specifically target AChE and BuChE. SC weights were significantly lower at P10 and P21 in hypothyroid pups but unchanged in the hyperthyroid ones. Hypothyroidism significantly reduced both *specific and total* AChE activity in SC of P10 and P21 rat pups, while having no effects on the BuChE activity, although total BuChE activity was decreased due to reduced total tissue weight. In contrast both specific and total AChE activities were markedly and significantly *increased* (>100%) in the P10 and P21 hyperthyroid pups. However, BuChE specific activity was unaffected by this treatment. The results indicate that hypothyroid condition significantly reduces, while hyperthyroidism increases, the postnatal development of cholinergic synapses, thereby influencing the functional development of this major sensory and motor structure. However, the neurochemical development of glia and other non-neuronal cells, where BuChE is mainly localized, is comparatively unaffected in these abnormal developmental conditions.

1. Introduction

Thyroid hormones (TH) have profound effects on the developing central nervous system of mammals (see Eayrs, 1971; Farahvar and Meisami, 2007; Lauder, 1977; Legrand, 1982; Meisami, 1983; Timiras, 1988 for reviews of early and later studies). TH deficiency in human infants results in cretinism, which involves severe neurological defects and mental retardation. Due to such drastic effects, most experimental investigations have focused on the effects of TH on the development of brain and its parts, particularly in altricial rodents such as the rat that are born with a fairly immature nervous systems and, in whom TH secretion develops mostly postnatally. In the present study, we therefore investigated the effects of excess and deficiency of TH on the postnatal neurochemical development of the spinal cord (SC) as this region of the CNS is relatively less investigated, compared to the brain.

Specifically, we focused on the development of the activity of the cholinergic enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in the SC of developing postnatal preweaning rat pups. Central neural AChE is a membrane-bound enzyme localized mainly in cholinergic neurons and their synaptic

Abbreviations: AChE, acetylcholinesterase; ASChCl, acetylthiocholine chloride; bw, body weight; BuChE, butyrylcholinesterase = pseudocholinesterase; CNS, central nervous system; NS, not significant; P, postnatal; SC, spinal cord; TH, thyroid hormone(s); wt, weight.

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membranes, while BuChE is a cytoplasmic enzyme found mostly in the glial and vascular cells of the neural tissue (Barth and Ghandour, 1983; Bhatt and Tewari, 1978; Burt, 1975; Massoulie et al., 1993; Meisami, 1984; Meisami and Timiras, 1982; Nemat-Gorgani and Meisami, 1978). The neural tissue AChE, particularly the brain AChE (Abreu-Villaça et al., 2011), has been extensively studied and has been shown to play critical roles in peripheral and central nervous synapses. The development of AChE in the SC tissue occurs very similarly to that of the acetylcholine synthesizing enzyme choline-acetyl-transferase (ChAT) which is located mainly in cholinergic neurons and their synapses (Abreu-Villaça et al., 2011; Barber et al., 1984; Burt, 1975). AChE, like ChAT is therefore considered as marker for cholinergic neurons (Burt, 1975; Massoulie et al., 1993; Meisami, 1984; Meisami and Timiras, 1982; Nemat-Gorgani and Meisami, 1978).

Our choice of the SC as the experimental tissue for this study was based on the fact that it is a CNS structure rich in cholinergic neurons and their associated circuits and synapses (Barber et al., 1984). Motor neurons of the SC, both alpha and gamma types, are cholinergic, as well as some of their associated interneurons (Mesnage et al., 2011; Miles et al., 2007) and the preganglionic sympathetic motor neurons. Furthermore SC undergoes marked growth and development during the postnatal period, especially in altricial mammals such as the rat and its growth and differentiation continues beyond weaning, into young adulthood (Koohestani et al., in press; Meisami, 1983; Sousa and Horrocks, 1979).

Previous studies on the effects of TH on the developing CNS have largely focused on developmental hypothyroidism, in an effort to find an experimental model, analogous to the serious and debilitating human disease, cretinism. As a result, experimental *hyperthyroidism* has been less investigated, even though it too involves abnormal neurological effects (Ahmed et al., 2008; Timiras, 1988). We therefore investigated the effects of both hypo- and hyperthyroid conditions on cholinergic synaptic development of the SC to better assess TH effects on the development of this functionally important CNS structure. The results may help increase knowledge on the relative influence of TH, in terms of both their deficiency and excess, on both cholinergic neurons and their synapses, which express AChE, as well as and glial cells and other non-neuronal cells of neural tissue, which contain BuChE.

In a companion paper (Koohestani et al., in press) we also present data on the effects of *growth hormone* deficiency on postnatal changes in AChE and BuChE in the rat SC and have noted that growth hormone deprivation reduces AChE activity during the early and late postnatal periods, but does not alter BuChE specific activity. We believe that these findings not only enhance our knowledge of the hormonal influences on the development of the evolutionarily lower parts of the CNS, but also provide a more detailed understanding of the developmental causes of human cretinism and its neurological anomalies.

2. Materials and methods

2.1. Experimental animals and housing conditions

Pregnant (late-term) Sprague–Dawley dams (Harlan, Wisconsin) were housed in one of the animal colonies at the University of Illinois at Urbana-Champaign, and reared under normal room temperature and 12-hr light–dark cycles, with food and water provided ad libitum. The day of birth was considered as P1. After birth, male and female pups were culled down to 8 pups per dam per cage; runts and very low weight pups were removed so that only 8 healthy male and female pups remained per dam, in plastic cages. At the time of sacrifice, in order to avoid any effect of sex differences on the parameters being measured and the effects of treatments, only male pups were used for the SC growth and enzymatic assays. To assess normal growth and development, pups were observed carefully and weighed regularly. Animal care followed guidelines approved by the University of Illinois Division of Animal Resources and the University's "Institutional Animal Care and Use Committee (IACUC)", as well as those of the National Institutes of Health.

2.2. Hypo- and hyperthyroidism in growing rat pups

Following our previously published procedures (Farahvar and Meisami, 2007; Paternostro and Meisami, 1993; Tamasy et al., 1986a,b), developmental *hypothyroidism* was induced by adding the antithyroid goitrogen, propylthiouracil (PTU, 6-n-propyl-2-thiouracil; Sigma, St. Louis) to the drinking water of the litter at a concentration of 0.1% (w/v), from birth till the days of sacrifice (P1, P5, P10 and P21). On the recommendation of the University of Illinois veterinarian and following the practice described in our previous publications (Farahvar and Meisami, 2007; Paternostro and Meisami, 1993; Tamasy et al., 1986a,b), a small amount of a non-caloric sweetener (saccharin) was added to the water bottles of the hypothyroid litters, at the concentration of 0.01 mg/L, to mask the bitter taste of PTU and prevent reduced water intake. Our previous studies have shown that PTU administration plus the artificial sweetener results in adequate water intake, decreased plasma T_4 levels by 100% and a several-fold increase in plasma TSH levels (Farahvar and Meisami, 2007; Paternostro and Meisami, 1991, 1993; Tamasy et al., 1986a,b).

To induce developmental *hyperthyroidism*, a solution of L-thyroxine (T_4) sodium salt penthydrate [Sigma, 0.005 ml/g body wt. of a stock solution (60 μg T_4/ml of phosphate buffer solution = PBS), pH 9–10)], was injected subcutaneously to pups daily from birth to P21 (weaning) according to our previously published procedure (Paternostro and Meisami, 1991). Body weights and appearance of developmental landmarks (ear and eye opening, changes in facies, fur growth, mobility, etc.) were checked for hypo- and hyperthyroid conditions, per our previous procedures and reports (Paternostro and Meisami, 1991, 1993; Farahvar and Meisami, 2007).

2.3. Tissue collection

Male pups at the ages of P1, P5, P10 and P21 (weaning) were selected and anesthetized with halothane via a vaporizer, decapitated, and the SC tissue was collected by the 'toothpaste tube' method as described in Sousa and Horrocks (1979) and Meisami (1983). The vertebral column was cut away from the surrounding tissues longitudinally; two pairs of pliers were pressed on the vertebral column consecutively, moving caudocranially, resulting in extrusion of SC tissue from the anterior opening of vertebral column. Collected SC tissue samples which included all parts of the SC from sacral to cervical regions, were weighed to the nearest mg and immediately frozen using liquid nitrogen and stored at −80 °C until used in enzyme assays.

2.4. Reagents

Acetylthiocholine chloride (ASChCl, Sigma, St. Louis) was used as the substrate and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) as the color reagent. The inhibitor substances BW284C51 and iso-OMPA (Sigma, St. Louis) were used as the specific inhibitors of AChE and BuChE respectively. Phosphate buffer solution (PBS) at 7 mM was a product of HyClone Company (Logan, UT). Double distilled water was used throughout the experiment.

2.5. Acetylcholinesterase and butyrylcholinesterase assays

The enzymatic assays for AChE and BuChE were carried out according to the spectrophotometric method of Ellman et al. (1961) as modified slightly by Nemat-Gorgani and Meisami (1978). Six SC tissue samples from 6 rat pups per age and experimental groups (control, hypo- and hyperthyroid) were utilized. The entire whole frozen SC tissue samples were weighed to the nearest mg and homogenized in 7 mM PBS at pH 8.0, using an Eberbach glass homogenizer, to reach a concentration of 20 mg/ml (2%, w/v). The final assay tube contained 0.4 ml tissue homogenate, 2.6 ml PBS buffer at pH 8.0, 0.1 ml DTNB (at pH 7.0), and 0.02 ml acetylthiocholine at pH 8.0.

In the AChE assay medium, a solution of the inhibitor substance iso-OMPA was added to the enzyme assay medium at a final concentration of 0.3 μM in order to inhibit BuChE activity. Similarly, in the BuChE assay medium, a solution of the inhibitor substance BW284C5 was added to the BuChE assay tubes at a final concentration of 6.0 μM, to inhibit AChE. Absorbance changes during the 10-minute assay were measured spectrophotometrically at 412 nm at 22 °C for both AChE and BuChE enzymes, respectively. Each assay was done in triplicate and optical density changes per minute were recorded and averaged. *Specific* and *total* activities of the enzymes were calculated and expressed as "*nmol substrate hydrolyzed/min/mg tissue*" and "*nmol substrate hydrolyzed/min/whole SC tissue wt.*".

2.6. Statistical analysis

AChE and BuChE activities were expressed as the group means ± standard errors of the means (mean ± S.E.M.) for each of the two enzymes per age and experimental group, as detailed above. Enzyme assays were performed in triplicates and each result represents the mean of at least three independent assays. To determine the significance of the differences between the means of the experimental vs. control groups at each age group, as well as for the significance of changes in the