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Postnatal growth hormone deficiency in growing rats causes marked decline in the activity of spinal cord acetylcholinesterase but not butyrylcholinesterase

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ABSTRACT

The effects of growth hormone (GH) deficiency on the developmental changes in the abundance and activity of cholinesterase enzymes were studied in the developing spinal cord (SC) of postnatal rats by measuring the specific activity of acetylcholinesterase (AChE), a marker for cholinergic neurons and their synaptic compartments, and butyrylcholinesterase (BuChE), a marker for glial cells and neurovascular cells. Specific activities of these two enzymes were measured in SC tissue of 21- and 90 day-old (P21, weaning age; P90, young adulthood) GH deficient spontaneous dwarf (SpDwf) mutant rats which lack anterior pituitary and circulating plasma GH, and were compared with SC tissue of normal age-matched control animals. Assays were carried out for AChE and BuChE activity in the presence of their specific chemical inhibitors, BW284C51 and iso-OMPA, respectively. Results revealed that mean AChE activity was markedly and significantly reduced [28% at P21, 49% at P90, ($p < 0.01$)] in the SC of GH deficient rats compared to age-matched controls. GH deficiency had a higher and more significant effect on AChE activity of the older (P90) rats than the younger ones (P21) ones. In contrast, BuChE activity in SC showed no significant changes in GH deficient rats at either of the two ages studied. Results imply that, in the absence of pituitary GH, the postnatal proliferation of cholinergic synapses in the rat SC, a CNS structure, where AChE activity is abundant, is markedly reduced during both the pre- and postweaning periods; more so in the postweaning than preweaning ages. In contrast, the absence of any effects on BuChE activity implies that GH does not affect the development of non-neuronal elements, e.g., glia, as much as the neuronal and synaptic compartments of the developing rat SC.

1. Introduction

Growth hormone (GH), a peptide hormone synthesized and secreted by the somatotrope cells of the anterior pituitary gland, is well-known to play major roles in growth and development of mammalian tissues, particularly bone and muscle. However, in contrast to thyroid hormones, which have profound effects on developing neural tissue and the central nervous system of mammals (Balãzs, 1971; Eayrs, 1971; Farahvar and Meisami, 2007; Koohestani et al., 2012; Legrand, 1982; Nunez, 1984; Paternostro and Meisami, 1993, 1996; Timiras, 1988), GH has been widely thought to have less or no influence on CNS development. The latter belief is based on the pioneering studies of Herbert Evans and collaborators in the early 1950s (Walker et al., 1950, 1952) and Diamond and co-workers in the late 1960s (Diamond, 1968; Diamond et al., 1969; Gregory and Diamond, 1968a,b), which revealed that neonatal hypophysectomy in 6-days old rat pups, which removed the source of systemic GH, did not inhibit brain growth and morphology, while concomitantly inhibited the skeletal and body growth markedly. Similarly, GH injections in hypophysectomized rats did not stimulate brain growth but markedly promoted body and skeletal growth (Diamond, 1968; Diamond et al., 1969; Gregory and Diamond, 1968a; Walker et al., 1950, 1952).

In the present study we investigated the effect of GH absence on the postnatal developmental changes in activity of the cholinergic enzymes *acetylcholinesterase* (AChE) and *butyrylcholinesterase* (BuChE). Cholinergic enzymes have been widely investigated in neural tissue and have been shown to play extremely critical roles in the neuronal activity of the peripheral and central nervous systems (Abreu-Villaça et al., 2011; Massoulie et al., 1993; Meisami, 1983). The SC is a CNS structure with abundant cholinergic neurons and synapses (Burt, 1975; Meisami, 1983). AChE, a membrane bound enzyme of the neuronal synapses (Meisami, 1984; Nemat-Gorgani and Meisami, 1978), is considered a specific neurochemical

Abbreviations: AChE, acetylcholinesterase; ASChCl, acetylthiocholine chloride; BuChE, butyrylcholinesterase; ChAT, Choline acetyltransferase; ChE, Cholinesterase; CNS, central nervous system; GH, growth hormone; NS, not significant; P, postnatal; SC, spinal cord; *SpDwf*, spontaneous dwarf mutant rats.

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marker of cholinergic neurons, particularly of their synaptic (post-synaptic) membranes. In contrast, BuChE is known to be cytosolic (Meisami, 1984) and is found in the glial cells and cells of the blood vessels of neural tissue. BuChE does not participate in synaptic functions but takes part in various aspects of choline metabolism in neural tissues (Barth and Ghandour, 1983; Bhatt and Tewari, 1978; Massoulie et al., 1993; Meisami, 1984; Nemat-Gorgani and Meisami, 1978).

Earlier investigators studying the effects of GH effects on the developing mammalian tissues used neonatally hypophysectomized rats that become GH deficient (Walker et al., 1950, 1952; Diamond, 1968; Diamond et al., 1969). However, those studies focused on the brain but not the SC. Because our goal was to investigate the effects of GH deficiency on the development of cholinergic enzymes activities, we focused our investigation on the SC, which is very rich in cholinergic neurons and cholinergic synapses. In the present study, however, we utilized postnatal mutant rats (*Spontaneous dwarf mutant*, *SpDwf*) that lack GH and are therefore markedly retarded in their somatic growth (Larsen et al., 2005; Nogami et al., 1989) in order to determine if cholinergic neurochemical development in their SC was also adversely affected. The value of using GH-deficient over hypophysectomy is that the former allows us to study deficiency of a single hormone (GH here), while the former results in deficiency of several other tropic hormones, which must be replaced to observe the effects of GH exclusively.

Specifically, we compared the activities of the cholinergic enzymes (AChE and BuChE) in the SC of the SpDwf mutants that lack GH in both pituitary gland and plasma, with those of the age-matched normal control rats of the Sprague-Dawley strain. To explore possible effects of other hormones regulating growth and neurochemical development of SC, we have also studied the effects of the deficiency and excess of *thyroid hormones* on the postnatal development of AChE and BuChE enzymes in this same CNS region (spinal cord) of the developing rat (Koohestani et al., 2012).

2. Materials and methods

2.1. Control and experimental animals

Rats of the Sprague-Dawley strain bred in the animal colony of the University of Illinois, Urbana-Champaign, were utilized for normal control animals. Experimental GH deficient P21 and P90 *SpDwf* (*Spontaneous dwarf*) mutant rats utilized in this study were a gift from the laboratory of Prof. Phil Best at the University of Illinois, Urbana-Champaign. The *SpDwf* mutant rats (initially identified as "SDR" or "spontaneous dwarf rats") arose from the original normal Sprague-Dawley strain of rats and have been widely used in experimental studies involving laboratory rats. The *SpDwf* rats are a mutant strain lacking GH in both the anterior pituitary gland and blood plasma. Nogami et al. (1989) have detailed the genetic, cytogenetic and cytochemical abnormalities in the body and pituitary glands of GH deficient *SpDwf* rats. The absence of pituitary and circulating GH is the result of an autosomal recessive mutation in the GH gene in the somatotrope cells of the anterior pituitary gland, which produces an abnormal splice variant resulting in premature translational termination (see Nogami et al., 1989 for further cytological and molecular details). As a result, GH protein is not synthesized and its absence in both the somatotrope cells and blood plasma has been verified by various immunologic and immunocytochemical methods (Larsen et al., 2005).

Control and experimental (mutant) animals were housed and cared for under identical animal colony conditions in approved animal care facilities of the Division of Animal Resources of the University of Illinois. The housing and care conditions followed guidelines established by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois as well as guidelines of the National Institutes of Health. Specifically, lighting conditions were 12-h light–dark cycles and food (rat chow) and water were provided ad libitum for both groups.

After birth, male and female pups from each rat dam were reduced in number to 8 pups per dam per cage by removing the runts and low weight pups while retaining the healthy male and female pups. The litters were kept with their respective dams in approved plastic cages until weaning at P21, after which time pups were separated from their mothers and housed 4 male pups per cage. In the experiments presented in this paper, only males were chosen for weight measures and enzymatic assays, in order to have more homogenous and uniform sample size and avoid problems relating to sex differences. Both control and experimental animals were provided with high quality rat chow from birth until completion of the experiment (P90). The postweaning pups of both control and experimental groups appeared to consume their rat chow continuously and properly at normal levels although their food consumption was not quantified.

2.2. Tissue collection

Weanling and young adult rat pups at P21 and P90 were anesthetized with halothane via a vaporizer, decapitated, and whole 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 and removed from the body; two pairs of small surgical pliers were pressed on the vertebral column consecutively, moving caudocranially to facilitate removal of SC tissue from the vertebral column. This technique results in the extrusion of SC tissue from the anterior opening of the vertebral column. The extraction procedure was carefully carried out to ensure that all SC tissue in each animal was extruded and collected. However, we were less successful in achieving the same results in the case of GH deficient rats due to the softness and fragility of vertebral bone tissue. As a result, even though we were able to obtain substantial and possibly nearly all of the SC tissue from the GH deficient pups, and also because the full weights of the SC are not relevant to the parameters of the cholinergic enzyme specific activity measured here, we do not present any results regarding the full total weight of SC tissue in the control and experimental groups in the Results section of this paper. However, the issue of the effect of GH deficiency on the SC weight will be briefly raised in Section 4.

The number of animals used at each age and experimental group, whose tissue samples were used for enzymatic assays, included six weanling or young adult male rats per age and experimental group. Wet weights (to the nearest mg) were measured individually for each animal's collected SC tissue. The collected tissue from each pup was mixed to allow for the calculation of overall average specific activity of the two cholinergic enzymes within the SC tissue and eliminate any possible variation stemming from regional differences. The collected SC tissue included all parts of the SC from most caudal upward to cranial. After mixing and weighing, the individual SC tissues (per rat) were immediately frozen using liquid nitrogen and stored at −80 °C until used for enzymatic assays.

2.3. 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 cholinesterase inhibitors BW284C51 and iso-OMPA (Sigma, St. Louis) were used as the specific inhibitors of AChE and BuChE respectively. Phosphate buffered saline (PBS) at 7 mM was a product of HyClone Company (Logan, Utah). Double distilled water was used throughout the experiment.

2.4. Acetylcholinesterase and butyrylcholinesterase assays

The enzymatic assays for AChE, BuChE and total ChE were carried out by the method of Ellman et al., 1961 as modified by Nemat-Gorgani and Meisami, 1978 (see also Meisami, 1983, 1984). Whole SC tissue samples from six animals per age group (P21 and P90) and condition (control and experimental groups) were utilized for enzymatic assay analysis. 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 of tissue (2%, w/v). The final assay tube contained 0.4 ml tissue homogenate, 2.6 ml PBS buffer (pH 8.0), 0.1 ml DTNB (pH 7.0), and 0.02 ml acetylthiocholine (pH 8.0).

To inhibit BuChE, an inhibitor solution of iso-OMPA at a final concentration of 0.3 μM was added to the enzyme assay medium; similarly to inhibit AChE, an inhibitor solution of BW284C5 at a final concentration of 6.0 μM was added to the AChE assay tubes. Absorbance changes were measured during the 10-min enzymatic assays by a spectrophotometer at 412 nm at room temperature (22 °C). Each assay was done in triplicate and optical density changes per minute were averaged. For each control and experimental group, enzymatic assays were carried out once in the presence of substrate and BW284C51 for *AChE specific activity* and another time in the presence of substrate and iso-OMPA for *BuChE specific activity*. Enzyme specific activities were calculated and expressed as "*nmoles substrate hydrolyzed/min/mg SC tissue*" as described previously (Ellman et al., 1961; Meisami, 1983, 1984).

2.5. Statistical analysis

AChE and BuChE activities were expressed as the mean and standard error of the mean (mean ± S.E.M.). Enzyme assays were performed in triplicates and each result represents the mean of at least three independent assays. To determine the significant differences between the results of the experimental vs. control groups at P21 and P90 as well as across the two ages, the data were subjected to statistical analysis using the statistical software STATA (Version 11) which permits statistical analysis using the *t*-test for comparison between two groups (in our case, unpaired sample means) and ANOVA (analysis of variance) for comparisons across more than one group and also for interaction among the various groups. The ANOVA test specifically ensured appropriate statistical comparison across both treatment and age groups. The results on mean body weight and activity of AChE in SC, as reported below in detail, confirmed highly significant differences between body weight and AChE