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Expression and p75 neurotrophin receptor dependence of cholesterol synthetic enzymes in adult mouse brain

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Abstract

Normal brain function depends critically on cholesterol. Although cholesterol is synthesized locally in the adult brain, the precise anatomical localization of cholesterogenic enzymes is not known. Here we show that 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAred) and 7-dehydrocholesterol reductase (7dhcred), the first and last enzymes, respectively, in the cholesterol biosynthesis pathway, are co-expressed in neurons throughout adult murine brain. Co-localization is most prominent in cortical, hippocampal, and cholinergic neurons. Since adult hippocampal and cholinoergic neurons express p75 neurotrophin receptors (p75NTR) we hypothesized that p75NTR regulates expression of cholesterogenic enzymes. Treatment of Neuro2a neuroblastoma cells or primary cerebellar cultures with siRNA downregulates p75NTR and decreases the expression level of HMG-CoAred and 7dhcred. Native neuroblastoma cell lines with differential expression of p75NTR differentially express 7dhcred; 7dhcred expression correlates with p75NTR expression. This suggests that, in p75NTR-expressing cells, p75NTR regulates cholesterol synthesis through regulation of HMG-CoAred and 7dhcred expression. The unexpected localization of cholesterogenic enzymes in adult neurons suggests that at least some adult neurons retain the ability to synthesize cholesterol.

Keywords: P75 neurotrophin receptor; Cholesterol; HMG-CoAred; 7dhcred; In situ hybridization

1. Introduction

Cholesterol makes up 20–30% of all lipids in the brain [6]. It is an essential membrane component [26], and serves as a precursor or cofactor for signaling molecules and neurotransmitter release [25]. Cholesterol is synthesized in the brain [15]. Its synthetic rate plateaus in the 1st week of postnatal life and remains constant thereafter [32,48]. The respective roles of neurons and glial cells in cholesterol biosynthesis in brain are not known [39]. Furthermore, although embryonic neurons synthesize cholesterol, it is not clear whether adult neurons continue to do so, given the relatively reduced demand for "new" cholesterol in mature brain [48]. In fact, it has been hypothesized that regional cholesterol synthesis and homeostasis differs between developing and mature central neurons [39].

The rate-limiting enzyme in cholesterol biosynthesis is 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAred; EC 2.3.3.10) [23]. This enzyme catalyzes conversion of HMG-CoA to mevalonic acid, derivatives of which play roles in the synthesis of several biologically important compounds, including carotenoids, ubiquinone, Vitamins E and K, and squalene [18]. Cholesterol biosynthesis begins with formation of squalene and ends with the reduction of 7-dehydrocholesterol [10]. This final step is catalyzed by 7-dehydrocholesterol reductase (7dhcred; EC 1.3.1.21) [21]. Partial or complete lack of 7dhcred enzymatic activity leads to severe developmental malformations and mental retardation, as seen in patients with Smith–Lemli–Opitz syndrome [14,31,34,45]. Despite the pivotal role of cholesterol in the central nervous system, relatively little is known about its

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homeostasis. A detailed expression map of cholesterogenic enzymes and their regulation in the adult nervous system is lacking.

In the present study, we analyzed the regional expression of HMG-CoAred and 7dhcred in adult mouse brain. We found that both enzymes are expressed in neurons throughout the brain. Using double *in situ* hybridization we show that cortical, hippocampal, and cholinergic neurons contain both enzymes. Interestingly, in the cells that express p75NTR, the expression level of p75NTR appears to modulate the expression level of HMG-CoAred and 7dhcred. This provides evidence that adult neurons contain the enzymatic machinery for cholesterol biosynthesis and suggests that, at least in a pathologically important subset of central neurons, this machinery may be modulated by p75NTR expression.

2. Materials and methods

2.1. In situ hybridization

Cryostat sections (20 μm thick) were prepared from fresh frozen brain specimens stored at −80 °C. Analyses were performed on coronal sections obtained from three adult mice at 4 months of age. Two sections were mounted on each Superfrost Plus slide, air dried, and again stored at −80 °C until required. The p75NTR intracellular domain (p75ICD) probe was generated using forward (gtggaacagctgcaaacaaa) and reverse (cgctgcatagactctccaca) primers and FLp75 plasmid DNA as a template [30]. The HMG-CoAred probe was generated using forward (ccagtaacccaaagggtcaa) and reverse (gatcctcctccactgacgaa) primers and mouse brain cDNA as a template. The 7dhcred probe was generated using forward (accattgacatctgccatga) and reverse (cggtgggtaagcaggatagt) primers and mouse brain cDNA as a template. In all cases, the PCR products were TA-cloned into a plasmid that has T7 and SP6 sites (pSTBlue1, Novagen). The plasmids were sequenced to confirm successful cloning. SP6 and T7 polymerases were used to synthesize antisense and sense ^{35}S-labeled riboprobes from the cloned PCR products: antisense riboprobe to detect mRNA for the gene of interest and sense probe as negative control. During hybridization, approximately 3 ng of each probe ($\sim 2 \times 10^6$ CPM) were used per slide in a total volume of 90 μl. These methods were described previously [30]. Following hybridization and washing, slides were air dried and exposed to BioMax MR film (Kodak) for 8–90 h and then dipped in emulsion (NTB2, Eastman Kodak Company, Rochester, NY), and exposed for 3 days to 3 weeks at 4 °C. After appropriate exposure time, the slides were developed and lightly counterstained with toluidine blue. High-resolution scans of each film image and darkfield images were captured from the developed slides. While antisense HMG-CoAred, 7dhcred, and p75ICD probes gave specific signals, the corresponding sense probes did not produce any labeling.

For double *in situ* hybridization, p75ICD, HMG-CoAred, and 7dhcred probes were labeled using DIG (digoxigenin) RNA labeling mix following the manufacturer's recommendations (Roche Diagnostics GmbH, Indianapolis, IN). The procedure for double *in situ* labeling was similar to the one described for the radioactive probe with slight modifications [8]. Both DIG and ^{35}S probes were hybridized at the same time. After washing, the slides were incubated with anti-digoxigenin-AP antibody (Roche Diagnostics Corporation, Indianapolis, IN), washed, dried and exposed to X-ray film. Next the slides were dipped in autoradiography emulsion (NTB2, Eastman Kodak Company, Rochester, NY) for a week, developed, and then applied NBT/BCIP solution (Roche Diagnostics Corporation, Indianapolis, IN) to develop DIG-labeling. The slides were covered with crystal/mount mounting solution (Biomeda, Foster City, CA) and examined under the microscope. Prior to double *in situ* hybridization, each DIG-labeled probe was applied individually to control sections to determine the optimal amount of probe and confirm the specificity of the probes. We initially compared DIG-HMG-CoAred and ^{35}S-labeled-7dhcred, and DIG-7dhcred and ^{35}S-labeled-HMG-CoAred. The best visualization was obtained with DIG-HMG-CoAred and ^{35}S-labeled-7dhcred. HMG-CoAred is expressed at higher levels than 7dhcred (1 day X-ray film exposure for HMG-CoAred riboprobe corresponds to 3.5 days of X-ray film exposure for 7dhcred). Since DIG labeling gives a weaker signal than ^{35}S-labeled riboprobes, the best results for double *in situ* hybridization are obtained when DIG label is used with the "high expressor" and ^{35}S-label with "weak expressor". Similarly with probes for other mRNAs, analogous combinations gave excellent results (e.g., DIG-p75ICD with ^{35}S-labeled-7dhcred or ^{35}S-labeled-HMG-CoAred riboprobes gave excellent visualization because p75NTR is expressed at a very high level in cholinergic neurons).

2.2. Animals

C57BL/6J mice (stock number 000664) were obtained from Jackson Laboratory (Bar Harbor, ME). The use of animals in this study was approved by the IACUC of the Children's Hospital of Pittsburgh.

2.3. Cell cultures (neuroblastoma cell lines and primary cerebellar neurons) and siRNA transfections

Neuroblastoma cell lines were purchased from American Type Culture Collection (Rockville, MD). Neuro2a, SK-N-BE(2), SK-N-SH, IMR-32, and CHP-212 cells were maintained in the alpha modification of minimal essential medium (Eagle) with Earle's salt, and supplemented with L-glutamine, sodium bicarbonate, non-essential amino acids, sodium pyruvate, and fetal bovine serum. All cells were subcultured once a week, and the culture medium was changed every 3 days.

Neuro2a cells were cultured for 2 days before transfections. Cells were transfected with each of five different