Behavioural Brain Research 206 (2010) 101–108

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Dysfunction of muscarinic acetylcholine receptors as a substantial basis for progressive neurological deterioration in GM3-only mice

Orie Tajima[a,c], Nobuaki Egashira[d,e], Yuhsuke Ohmi[a], Yoshihiko Fukue[d], Kenichi Mishima[d], Katsunori Iwasaki[d], Michihiro Fujiwara[d], Yasuo Sugiura[b], Keiko Furukawa[a,c], Koichi Furukawa[a,*]

[a] Department of Biochemistry II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan
[b] Department of Anatomy II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan
[c] Department of Biomedical Sciences, Chubu University College of Life and Health Sciences, 1200 Matsumoto, Kasugai 487-8501, Japan
[d] Department of Neuropharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka 814-0180, Japan
[e] Department of Pharmacy, Kyushu University Hospital, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 27 July 2009
Received in revised form 26 August 2009
Accepted 2 September 2009
Available online 8 September 2009

Keywords:
Ganglioside
Knockout
Behavior
Memory
Learning
Acetylcholine receptor
Degeneration

ABSTRACT

To examine the effects of deletion of gangliosides on the nervous system by avoiding masking effects with the remaining structures, we established double knockout (DKO) mice of GM2/GD2 synthase and GD3 synthase genes, i.e., GM3-only mice. They showed progressive sensory and motor neuron deficits with aging. We further examined higher order neurological functions, and found progressive dysfunction of motor coordination with rota-rod test and marked deterioration in memory and learning with eight-arm radial maze test in the DKO mice. The results of oxotremorine treatment suggested that they undergo strong suppression of muscarinic type acetylcholine receptors (mAChRs) functions, and that the damage in the GM3-only mice is due to a mAChR receptor deficit. On the other hand, expression levels of mRNAs of mAChRs were generally up-regulated, suggesting compensatory increase of expression due to reduced functions. Since central mAChRs are involved in the regulation of cognitive, behavioral, sensory, motor, and autonomic functions, we investigated changes in the expressions levels of subtypes of the mAChR genes in various regions of brain tissues. M1 and M4 receptors were conspicuously up-regulated in cortex and striatum in the DKO, suggesting that suppressed functions of mAChRs are responsible for the altered neurological features, in particular for deteriorated memory and learning, observed in the behavioral analyses. Thus, dysfunction of mAChRs might be a substantial basis for the progressive neurological deterioration in DKO mice.

1. Introduction

Sialic acid-containing glycosphingolipids, gangliosides have been considered to play roles in the neuronal development and functions based on their strict-regulated spatio-temporal expression patterns in nervous systems [38], and on a number of experimental data with cultured cells and ganglioside-administrated animals. Recent studies with genetic manipulation of glycosyltransferases responsible for the synthesis of glycosphingolipids have demonstrated that main roles of gangliosides are to maintain the structures and functions of nervous systems, and to repair damaged nervous tissues [11]. Our studies of gene knockout (KO) mice of β-1,4-N-acetylgalactosaminyltransferase (GM2/GD2 synthase) [32] and those of α2,8-sialyltransferase (GD3 synthase) [23] clearly indicated this conclusion [11]. These KO mice, in particular of GM2/GD2 synthase showed progressive neurodegeneration with aging [30].

However, abnormal phenotypes of these KO mice were milder than expected based on the wide range of distribution of gangliosides and various findings observed in *in vitro* experiments [28]. Although complex ganglioside-lacking mice showed age-related dysfunctions in both sensory and motor neuronal systems [30], they could survive and enjoy sufficient terms of life as the wild type mice. These mild phenotypes seemed due to compensatory effects with remaining glycolipids in the individual KO mice [11]. Usually, precursor structures of deleted glycolipids with gene disruption showed marked accumulation, suggesting defects due to deleted glycolipids could be compensated with increased amounts of the precursor structures. Actually, pathological examination of the neurodegenerative tissues in GM2/GD2 synthase KO mice revealed marked gliosis, abnormal morphology of spines and vesicles in synapses, suggesting that neuroregeneration occurred following

* Corresponding author. Tel.: +81 52 744 2070; fax: +81 52 744 2069.
E-mail address: koichi@med.nagoya-u.ac.jp (K. Furukawa).

0166-4328/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2009.09.005

neurodegenerative changes to repair the damages due to the gene KO [30].

In order to examine the straightforward effects of deletion of gangliosides on the nervous system by avoiding masking effects with the remaining structures, we established double KO (DKO) mice of GM2/GD2 synthase and GD3 synthase genes (GM3-only mice). Except GM3, there are no ganglio-series gangliosides including asialo-series in the DKO mice. They showed more serious phenotypes than each single KO, i.e., refractory skin lesion and neurodegeneration emerged in an early stage of life, sudden death after 25 weeks from the birth [10], and progressive sensory and motor neuron deficits with aging [31].

It has been reported that gangliosides are effective in protecting cholinergic neurons from degeneration or in correcting cholinergic deficiencies of the brain [3,22]. Acetylcholine (ACh) mediated neural functions are essential in central nervous systems (CNS) and peripheral nervous systems. In particular, muscarinic type acetylcholine receptors (mAChRs) are widely distributed in the whole bodies, and play key roles in various aspects by regulating the activity of many important functions of the central and peripheral nervous system [36]. Peripheral mAChRs mediate the classical muscarinic actions of ACh on organs and tissues that are innervated by parasympathetic nerves [2]. Central mAChRs are involved in regulating a large number of cognitive, behavioral, sensory, motor, and autonomic functions [9]. mAChRs consist of five isoforms, M1, M2, M3, M4 and M5. Among them, M1, M3 and M5 are coupled with G-protein of Gq, and GM2 and G4 are coupled with Gi, participating in different classes of heterotrimeric G-proteins [36].

In this study, we further examined higher order neurological functions, and found marked deterioration in memory and learning in the DKO mice. Also, oxotremorine treatment revealed that the DKO mice undergo strong suppression of mAChR functions. Therefore, we investigated changes in the functions and expressions of the mAChR genes that might be responsible for the altered neurological features with focus on the relevance with ganglioside deficiency.

2. Materials and methods

2.1. Animals

We have established double knockout (DKO) mutant mice of GM2/GD2 synthase and GD3 synthase in which only GM3 among the ganglio-series gangliosides remained [12]. DKO mice were generated by mating heterozygous mutants of GM2/GD2 synthase [32] and knockout mice of the GD3 synthase gene [23]. Wild type (WT) and DKO mice at various ages from 10 to 60 weeks old were used for all examinations. These mice were generated using ES cells derived from (C57BL/6 × CBA) F1, and phenotypic differences of these mutants from those showing audiogenic seizure (derived from ES cells of 129^{SvEv} origin) [14] seemed due to the distinct genetic backgrounds as already described [12]. Mice were kept under standard laboratory conditions of 12 h light followed by 12 h darkness. All experiments were performed following the guidelines of the Nagoya University Committee on Animal Research and the Experimental Animal Care and Use Committee of Fukuoka University. When these guidelines were constructed, the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) were followed as well as the guideline from the Ministry of Education, Culture, Sports and Technology of Japan (MEXT).

2.2. Rota-rod test

Mice were placed on the rotating rod (3 cm diameter; Neuroscience Inc., Tokyo, Japan) with a non-skid surface and the latency to fall was measured for up to 2 min. The test was performed three times per day for 3 days. The rotating speed was increased in the order of 5, 10 and 15 rpm each day.

2.3. Eight-arm radial maze task

Each mouse was weighed daily and the quantity of food was individually adjusted (2.0–3.0 g per day) in order to maintain the weight of each mouse at 85–90% of the initial level [6]. The apparatus of the eight-arm radial maze task was set at a 50 cm site from the floor. The maze consisted of a central platform with 18 cm in diameter and of eight arms extending radially. Food cups for reinforcement were located near the end of each arm. Each mouse was placed on a platform at the middle of the eight-arm radial maze and allowed to move freely in the maze. A trial was continued until the mouse achieved to enter in all eight arms or 5 min elapsed. The task was performed with one trial per day for 15 days. Performance in each trial was assessed by three parameters, i.e., numbers of correct choices in the initial eight chosen arms, numbers of errors defined as choosing arms which had already been visited, and running time elapsed until the animal ate all eight pellets.

2.4. Water maze test

To examine spatial memory and learning, water maze test was performed with mice at age 12–16 weeks (younger) and 29 weeks (older). The swimming pool (150 cm in diameter and 45 cm depth) was filled with water at 23 °C. A platform, 12 cm in diameter and 30 cm in height, was present inside the tank, with its top being 0.5 cm below the surface of water. Each mouse underwent three trials per day for three consecutive days. The trial was started by placing mice by hands into the water facing to the wall of the pool. The platform was located in a constant position in the middle of one quadrant and its location was fixed for all mice. In each trial, the swimming time needed to reach the hidden platform was recorded, and the cut-off time was set as 120 s. If a mouse failed to find the platform within 120 s, the mouse was forced to stay on the platform for 20 s. Performance of the test animal in each trial was assessed by swimming time.

2.5. Oxotremorine-induced tremor

To examine the function of mAChRs, mice were injected subcutaneously with 1.0 or 0.3 mg/kg oxotremorine, a non-selective muscarinic receptor agonist. The responses to the oxotremorine treatment were assessed immediately before and after injection. Tremor was scored with four grades, i.e., (0) no abnormal behavior observed; (1) intermittent slight tremors; (2) occasional moderate tremors as well as intermittent slight tremor; (3) persistent moderate tremors; and (4) persistent severe tremors. Observation of the tremors was made at 5-min intervals starting 30 min after the oxotremorine treatment, and the intensity of the tremors was determined as the total score per each 10 min after administration of oxotremorine [7].

2.6. Real-time RT-PCR

Total RNA from brains and spinal cords of male mice aged 50–60 weeks were isolated using TRIzol™ according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Whole brain was further divided into the cerebral cortex, corpus striatum, hippocampus, diencephalons, midbrain, pons-medulla and cerebellum under a stereoscopic microscope. To synthesize cDNA, reverse transcription was performed using 4 μg of total RNA, oligo-dT primer, and SuperScript II RT™ (Invitrogen, Carlsbad, CA) in a total volume of 50 μl. Expression levels of mAChR mRNAs were analyzed by SYBR Green-based real-time quantitative RT-PCR using DNA Engine Opticon2™ (Bio-Rad Laboratories) and DyNAmo™ kit (Finnzymes, Espoo, Finland). Following the real-time RT-PCR with sequence-specific primers of each receptor, relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method [17], and values of expression levels of receptor mRNAs were normalized with internal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) examined in parallel. Primer sequences used for real-time RT-PCR are listed in Table 1.

2.7. Statistical analysis

Results of behavioral analysis were expressed as means ± SEM. The number of mice used in the experiment was as indicated in each figure. Relative expression levels of the receptor were presented as means ± SD. Differences between the WT and the DKO mice were analyzed by the unpaired *t*-test. A *P*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Gangliosides deleted in DKO mice and an experimental schedule for behavioral analyses

Synthetic pathways of gangliosides and changes in their composition in DKO mice are shown in Fig. 1A as reported in a previous paper [31]. The general time schedule for the behavioral analyses is presented in Fig. 1B (an example of the older mouse group).

3.2. Dysfunction of motor coordination in DKO mice

The rota-rod performance for motor coordination was carried out with mice at age 10–14 weeks (younger) and 27 weeks (older). There were no differences of latency to fall at each rotating speeds